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THE DEVELOPMENT OF THE AZYGOS VEINS IN THE ALBINO RAT

By H. BUTLER
School of Anatomy, University of Cambridge

Recorded accounts of the development of the azygos veins of the albino rat (*Mus norvegicus albinus*) are scanty and difficult to interpret owing to confusion in the terminology applied to embryonic veins. Reagan (1927) described the development of capillary vascular arches between the proximal parts of the dorsal aortic rami and their accompanying segmental veins. Longitudinal anastomoses between these arches form the azygos veins. According to Schneider (1938) the left azygos vein, from its junction with the left superior vena cava to the point of entry of the inferior hemi-azygos vein at the 10th or 11th intercostal space, is developed from the left postcardinal vein. The remainder of the left azygos vein and the inferior hemi-azygos vein are developed from the supracardinal plexus. This plexus was first described in the rat by Butler (1927). Thus the development followed a pattern similar to that described by Hochstetter (1893). This pattern has since been shown to be incorrect for man (Seib, 1934), pig (Sabin, 1915; Reagan, 1919) and cat (Huntington & McClure, 1907).

TERMINOLOGY

Huntington & McClure (1907) defined the supracardinal veins as bilaterally symmetrical venous channels developed from longitudinal anastomoses between the segmental veins, and situated dorso-medial to the postcardinal veins. Cranially they join the postcardinal veins near their termination, and caudally at the point of entry of the hind limb-bud veins. Each vein is divided into a thoracic and a lumbar portion. The terminal arch of the adult azygos vein develops from the cranial end of the postcardinal vein and the remainder from the thoracic supracardinal veins. The final development of this bilaterally symmetrical azygos venous system varies from species to species. With the exception of the rat and the golden hamster the right lumbar supracardinal vein becomes the pre-renal inferior cava, i.e. the portion of the adult inferior vena cava between the common iliac veins and the renal veins. The left lumbar supracardinal vein disappears entirely.

Huntington & McClure (1907, 1920) do not state the position of the supracardinal vein relative to the sympathetic trunk, but Reagan (1919) states that they placed this vein on the medial side of the sympathetic trunk. Reagan (1927) pointed out that the azygos vein lies medial to the sympathetic trunk and its peri-aortic branches, whereas the pre-renal inferior vena cava lies lateral to these nerves. Thus the supracardinal portion of the azygos vein could not be regarded as being homologous with the pre-renal inferior vena cava. He regarded the main part of the azygos vein as developing from the medial sympathetic venous lines which have the following characteristics:

(1) They lie ventro-medially to the sympathetic trunk and its peri-aortic branches.

(2) Their segmental tributaries drain dorso-medially with reference to the sympathetic trunks.

(3) They lie ventro-laterally to the dorsal aortic rami.

Reagan & Tribe (1927) and Reagan & Robinson (1927) described the lateral aortic plexus as a venous line extending from the mid-thoracic to the mid-sacral region and lying lateral to the aorta and to the sympathetic trunk and its peri-aortic branches. It is divided into:

(1) A cranial thoraco-lumbar portion which acts as a functional substitute for the segmental tributaries of the degenerating postcardinal veins until the medial sympathetic venous line is fully developed. It then disappears completely, but for a time it exists side by side with the medial sympathetic venous line in the caudal portion of the thorax but separated by the sympathetic trunk.

(2) A caudal portion called the para-ureteric vein. On the right side this vein becomes the pre-renal inferior vena cava, on the left side it normally disappears.

The relationship of these embryonic veins and their adult derivatives may be summarized thus:

(1) The right and left medial sympathetic venous lines are the same as the thoracic supracardinal veins and form most of the azygos veins.

(2) The thoraco-lumbar venous line is not represented by any part of the supracardinal veins. It is functional only for a short time in the embryo and then disappears completely.

(3) The para-ureteric vein is the same as the lumbar supracardinal vein, and on the right side it becomes the pre-renal inferior vena cava. The left vein normally disappears.

Dorsal to the aorta and medial to the dorsal aortic rami is a pair of longitudinal venous lines described by Strong (1927), and called the dorsal aortic plexus. Reagan & Robinson (1927) called these the subcentral venous lines. They are connected to each other and to the medial sympathetic venous lines by cross anastomoses. These cross anastomoses form the retro-aortic connexions between the right and left azygos veins. The caudal portions of these longitudinal venous lines form the medial azygos root of Seib (1934).

Two further venous lines have been described but, as yet, little is known about them. Gladstone (1929) figured in an 11 mm. (6th week) human embryo the precostal or lumbo-costal venous line which lies immediately ventral to the transverse processes of the vertebrae and dorsal to the thoraco-lumbar line. Seib (1934) postulated a lumbo-costal venous line as a result of the study of anomalies of the adult human azygos veins. It lies between the postcardinal vein and the thoraco-lumbar venous line and becomes the ascending lumbar vein.

In the following descriptions the term supracardinal vein will not be used and wherever possible the terminology introduced by later writers will be employed.

MATERIAL AND METHODS

Twenty-six rat embryos ranging from the 14th to the 20th day were used. The post-copulation age was estimated as follows. The oestrous cycle of isolated virgin albino rats was charted by daily examination of vaginal smears (Long & Evans, 1926).

A buck was introduced when a pro-oestrous smear was found and frequent vaginal smears were made until spermatozoa were found. The midpoint between the time of the last sperm-free pro-oestrous smear and the oestrous smear containing spermatozoa was taken as the approximate time of copulation.

Some embryos were fixed in Bouin's fluid, embedded in wax and sectioned serially at 8–15μ according to their size. Sections were stained with haematoxylin and erythrosin.

Other embryos were injected with indian ink according to the method described by Popoff (1894). In most cases the anterior cardinal vein close to the ear was used for the injection. In small embryos the injection was sometimes made directly into the heart or liver. When the injection was completed the embryo was immediately fixed in Carnoy's fluid, embedded in celloidin and sectioned serially at 60–150μ. Some were bulk-stained in Grenacher's carmine prior to sectioning and others were cut and mounted unstained. Details of age and length of the embryos is given in Table 1. The C.R. length was measured after fixation.

Table 1

			Serial sections (thickness in μ)			
				Uninjected		
Group	Age (days)	(hr.)	Serial no.	C.R. length (mm.)	Transverse	Sagittal	Injected transverse
1	13	9	30	7	—	—	60
	—		30.1	—	—	—	60
2	13	10	20	7	8	—	—
			20.1	—	—	8	—
			20.2	—	—	—	75
			20.3	—	—	—	75
			20.4	—	—	—	100
			20.5	—	8	—	—
3	14	15	28	10	12	—	—
			28.1	—	—	—	100
			28.2	—	—	—	100
			28.3	—	—	—	100
			28.4	—	12	—	—
			28.5	—	—	15	—
4	15	9	27	11	12	—	—
			27.1	—	—	—	100
			27.2	—	—	—	100
			27.3	—	—	—	100
			27.4	—	—	—	100
5	17	9	33	15	15	—	—
			33.1	—	15	—	—
			33.2	—	—	—	100
			33.3	—	—	—	100
			33.4	—	—	—	150
			33.5	—	—	—	150
6	19	9	35	16	15	—	—

OBSERVATIONS

(1) *Embryos of groups* 1 (13 *days* 9 *hr.*) *and* 2 (13 *days* 10 *hr.*)

The postcardinal veins are almost fully developed and the right vein has a slightly smaller diameter than the left. At this age the segmental veins are mainly concerned with draining the relatively large spinal cord (Evans, 1912). The 9th segmental vein, which will become the first intercostal vein, drains into the anterior cardinal vein and

all the segmental veins caudal to this drain into the postcardinal vein. The segmental veins pass ventro-lateral to the sympathetic cord before they join the cardinal veins. The junction of the anterior and posterior cardinal veins is at the level of the 10th segment on both sides. The iliac anastomosis is a plexiform capillary connexion between the caudal extremities of the postcardinal veins.

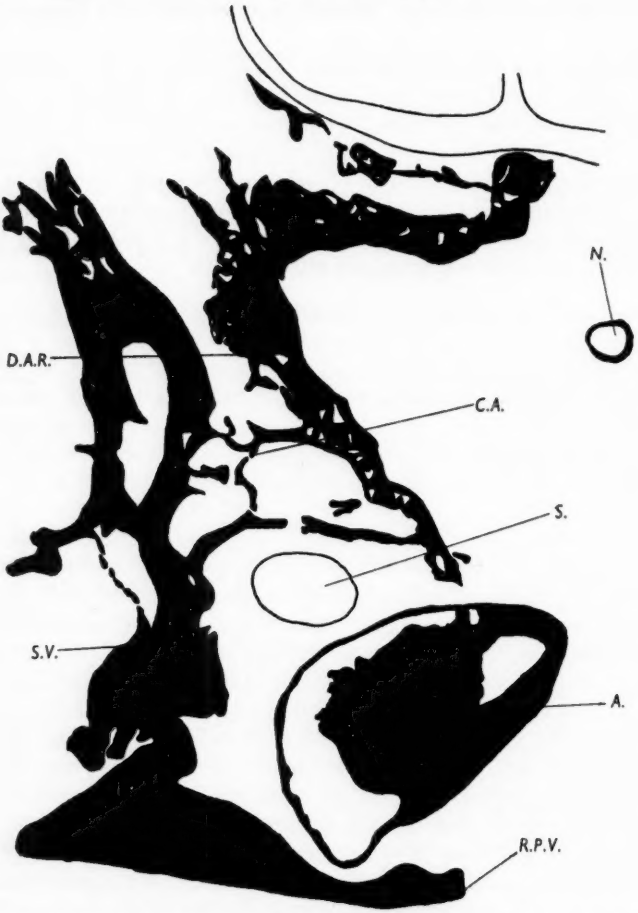

Fig. 1. Camera lucida drawing of a transverse section of embryo 30.1. Age 13 days 9 hr. C.R. length 7 mm. Vessels injected. ×200 (owing to reduction).

A new capillary plexus, more dorsally situated, develops at this stage. It commences as a series of vascular arches between the segmental veins and the accompanying dorsal aortic rami. The sympathetic cord lies within a triangle bounded by the stem of the dorsal aortic ramus, the stem of the segmental vein and the dorso-lateral aspect of the dorsal aorta (Fig. 1). Immediately dorsal to the sympathetic cord the artery and vein are in close proximity and it is here that the capillary arch

is formed. At first the arch is single, but it rapidly becomes plexiform and surrounds the sympathetic cord. Medial outgrowths from this perisympathetic plexus spread towards the mid-line dorsal to the aorta and form cross anastomoses. At this stage the perisympathetic plexuses are confined to the neighbourhood of the segmental vessels and there are no longitudinal anastomoses between them. The plexuses are first formed in the cervical region and their development proceeds caudally as far as the lumbar region.

The subcardinal veins are well developed and a plexiform intersubcardinal anastomosis is present. The connexion between the right subcardinal vein and the hepatic vein which will form the hepatic segment of the inferior vena cava is present as a capillary plexus.

Embryos of group 3 (14 *days* 15 *hr.*)

The inferior vena cava is well developed and consists of hepatic, subcardinal and postcardinal segments. The left subcardinal vein is still large. The iliac anastomosis is now a large channel. The body wall is well developed and the ribs are visible. The segmental veins are more concerned with the drainage of the body wall and so the segmental veins 9 to 21 may now be called intercostal veins 1–13. Portions of the postcardinal veins are beginning to disappear and certain intercostal veins have acquired new channels of drainage. The disappearance of the postcardinal veins commences at the level of the caudal postcardinal-subcardinal anastomosis and progresses in a cranial direction. It is more advanced on the left side than on the right.

The termination of the right intercostal veins in embryo no. 28.4 is:

(1) Intercostal vein 1 joins the anterior cardinal vein.

(2) Intercostal veins 2–5 join the right postcardinal vein passing ventro-lateral to the sympathetic cord.

(3) Intercostal veins 6–11 still join the postcardinal veins, but now have a second route of drainage into the perisympathetic plexus by a vessel passing dorsal to the sympathetic cord. This vessel corresponds to the dorsal basal anastomosis described by Reagan & Robinson (1927).

(4) Caudal to intercostal vein 11 the postcardinal vein has disappeared and the last two intercostal veins join the posterior aspect of the subcardinal segment of the inferior vena cava. They also drain into the perisympathetic plexus by dorsal basal anastomoses.

Between intercostal veins 6–12 there are indications of the formation of longitudinal anastomoses between the segmental perisympathetic plexuses. They are best developed caudally between intercostal veins 11 and 12. They lie on the dorsomedial aspect of the dorsal aortic rami in the position of the dorsal aortic plexus (Strong, 1927), or subcentral venous line (Reagan & Robinson, 1927) (Fig. 2).

The greater part of the left postcardinal vein has disappeared and has been replaced by a new longitudinal vein formed from longitudinal anastomoses between the segmental perisympathetic plexuses. The left intercostal veins terminate as follows:

(1) Intercostal veins 1 and 2 join the left anterior cardinal.

(2) Intercostal veins 3, 4 and 5 join the remaining part of the left postcardinal, passing ventral and lateral to the sympathetic cord.

(3) Intercostal veins 6–12 join the new longitudinal vein passing dorsal to the sympathetic cord via the dorsal basal anastomosis. The new longitudinal vein joins the remnant of the left postcardinal vein just caudal to intercostal vein 5, and terminates caudally by joining the cross anastomosis between the 12th pair of intercostal veins. It lies medial to the sympathetic cord and, except for its most caudal segment, it lies ventral and lateral to the dorsal aortic rami. Thus it is the medial sympathetic venous line (Reagan, 1927). The most caudal segment lies dorsal and medial to the dorsal aortic rami in the subcentral venous line. Examination of other embryos of this age and older shows that the new longitudinal vein on the left lies mainly in the medial sympathetic venous line, but occasional segments are in the subcentral venous line.

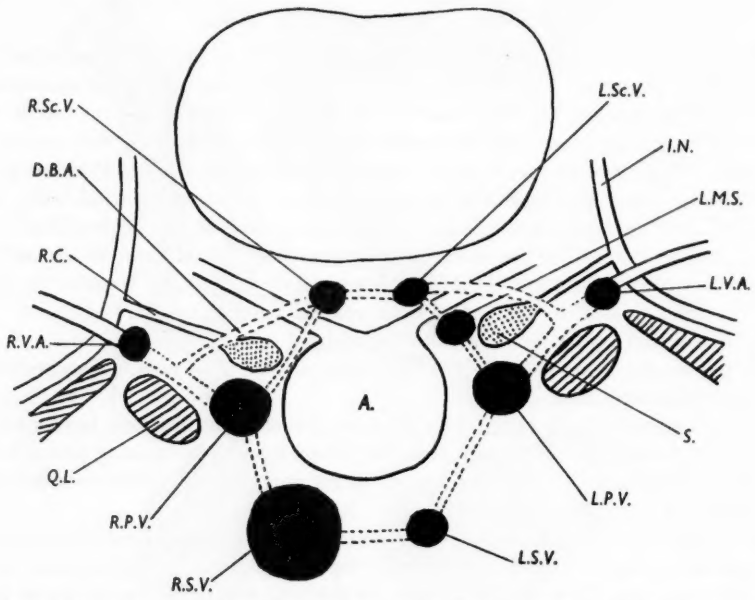

Fig. 2. Diagram to show the relationships of the longitudinal venous lines to the sympathetic cords and aorta.

(4) Intercostal vein 13 drains mainly into the posterior aspect of the left subcardinal vein and partly into the perisympathetic plexus (Fig. 3).

Litter-mates of this embryo showed a similar pattern with slight variations in the number of veins joining the subcardinal veins and in the extent of the new longitudinal veins.

(3) *Embryos of group* 4 (15 *days* 9 *hr.*)

The features of this stage are:

(1) Further disappearance of the right postcardinal veins.

(2) Extension of the longitudinal anastomoses in the right subcentral venous line.

(3) Disappearance of the intercostal drainage into the subcardinal veins.

The first right intercostal vein joins the right anterior cardinal vein. The right posterior cardinal vein extends from its junction with the anterior cardinal vein to the point of entry of intercostal vein 6. It receives intercostal veins 2–6. The remaining right intercostal veins pass dorsal to the right sympathetic cord and the aorta to join the new longitudinal vein developed in the left medial sympathetic venous line. The new portion of these veins which runs dorsal to the sympathetic cord and

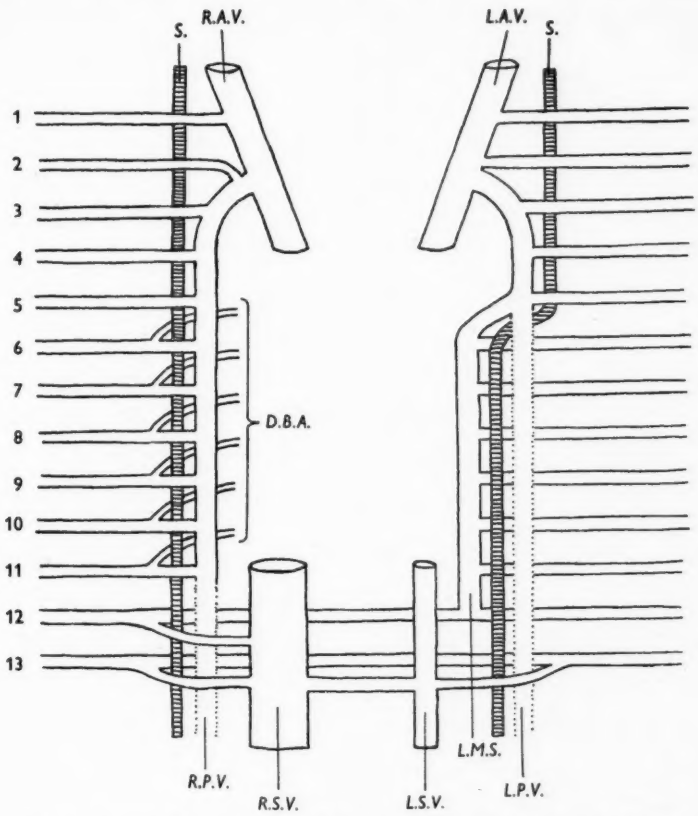

Fig. 3. Diagram of the venous lines in embryos of group 3. C.R. length, 10 mm.

aorta has developed from the perisympathetic plexus. Intercostal veins 6–13 are linked by a longitudinal anastomosis developed in the right subcentral venous line. This anastomosis is best developed in the caudal half of the thorax. The last two intercostal veins no longer join the right subcardinal vein.

The only change on the left side is that the last intercostal vein drains entirely into the perisympathetic plexus and has lost its connexion with the left subcardinal vein.

(4) *Embryos of group* 5 (17 *days* 9 *hr.*)

Caudal to intercostal vein 11, on both sides, the left medial sympathetic venous line and the right subcentral venous line become abruptly reduced in diameter. This is related to the formation of a new and more laterally situated longitudinal anastomotic vein between intercostal veins 11, 12 and 13. This new vein is situated:

(1) Along the lateral border of the quadratus lumborum muscle. The sympathetic cord lies along the medial border of this muscle.

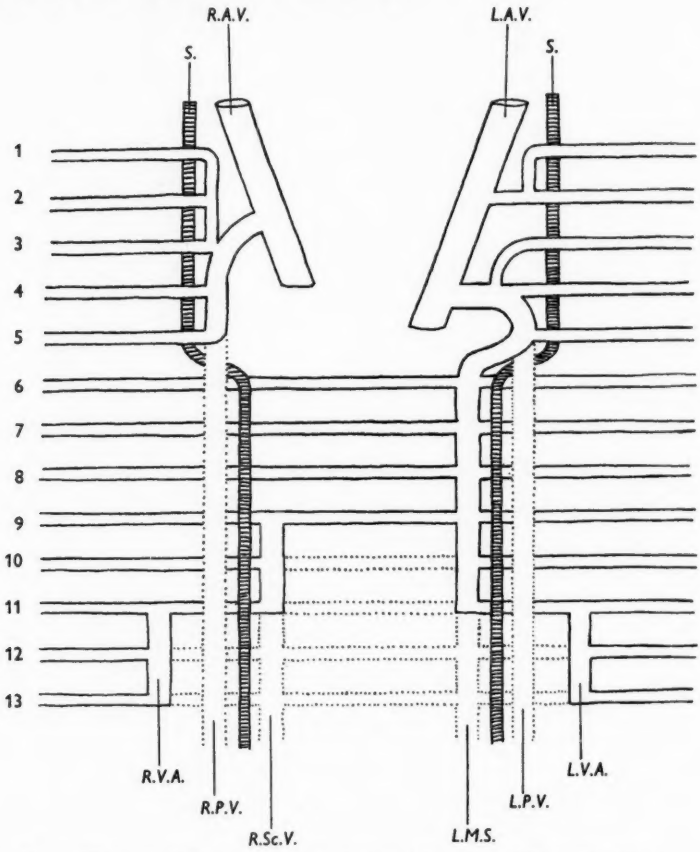

Fig. 4. Diagram of the venous lines in embryos of group 6. C.R. length, 16 mm.

(2) Ventral and lateral to the ramus communicans in the angle between the ramus communicans and the intercostal nerve.

(3) Either dorsal or ventral to the dorsal aortic ramus.

This new longitudinal vein will be referred to as venous line *A* (Figs. 2, 4 and 5). It receives the 12th and 13th intercostal veins and anastomoses with the left medial sympathetic and right subcentral venous lines respectively.

The retro-aortic anastomoses caudal to intercostal vein 9 have disappeared, and the right intercostal veins 9–13 join the left medial sympathetic venous line by a single enlarged retro-aortic cross anastomosis.

(5) *Embryos of group* 6 (19 *days* 9 *hr.*)

The adult pattern (Fig. 4) is now fully developed and consists of:

(1) The right superior intercostal vein receiving the first five intercostal veins and joining the right superior vena cava at the level of T. 3, passing medial to the phrenic nerve and lateral to the vagus nerve.

(2) Right intercostal veins 6–8 passing separately across the mid-line to join the left azygos vein and passing dorsal to the right sympathetic cord and aorta. Intercostal veins 9 and 10 may do likewise.

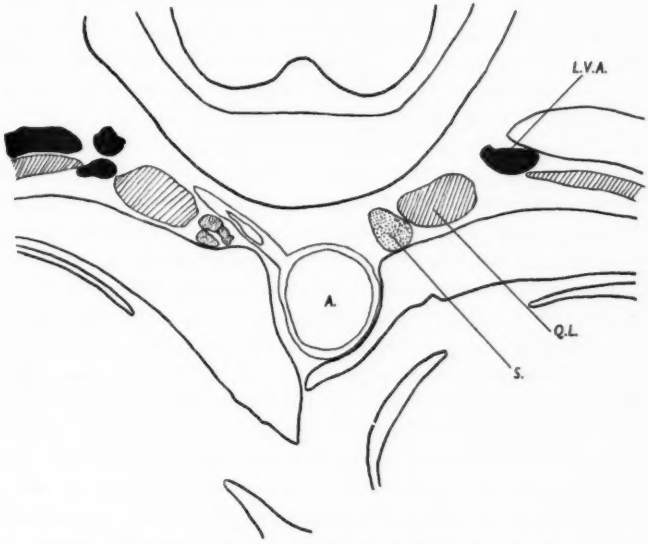

Fig. 5. Camera lucida drawing of a transverse section of embryo 33. Age 17 days 9 hr. C.R. length, 15 mm. Uninjected. ×55 (owing to reduction).

(3) The right azygos (inferior hemi-azygos) vein receiving the 9th to the 13th right intercostal veins and crossing the mid-line dorsal to the aorta and right sympathetic cord to join the left azygos vein.

(4) The left superior intercostal vein receiving the 1st and 2nd intercostal veins and then entering the left superior vena cava.

(5) The left azygos vein joining the left superior vena cava at the level of intercostal vein 4 and receiving left intercostal veins 3–13 and the inferior hemi-azygos vein. The terminal portion arches over the root of the left lung to join the left superior vena cava passing medial to the phrenic nerve and lateral to the vagus.

DISCUSSION

These observations show that only a small part of the azygos vein of the albino rat is derived from the postcardinal veins. The greater part of the azygos veins is developed from a more dorsally placed venous plexus which develops as the postcardinal veins disappear. The cranial end of the left azygos from its junction with the left superior vena cava to the point of entry of the 5th or 6th left intercostal vein is all that remains of the left postcardinal vein. The pattern of development is the same as that described for the pig (Reagan, 1919), and not as that described for all mammals by Hochstetter (1893) nor for the albino rat by Schneider (1938). Boyer's (1948) account of the development of the azygos vein of the golden hamster lacks sufficient detail for an accurate comparison but suggests that the pattern is similar.

The remainder of the left azygos vein, the inferior hemi-azygos vein and the retro-aortic portion of the 6th–8th right intercostal veins are developed from the peri-sympathetic plexuses. These plexuses begin as vascular arches between the dorsal aortic rami and the accompanying segmental veins in the embryos of groups 1 and 2 (7 mm. C.R. length). Subsequent developments of these capillary arches give rise to plexuses which surround the sympathetic cords and spread across the mid-line dorsal to the aorta. At this stage the rat embryo closely resembles the 13·5 mm. pig embryo described by Sabin (1915). Frazer (1931) described similar plexuses in the human embryo of about 8 mm. Longitudinal anastomoses grouped around the sympathetic cords and aorta link up the segmental perisympathetic plexuses (Fig. 2). Seib (1934) gives a full account of these longitudinal venous lines as seen in the human embryo. Only the right and left subcentral venous lines and the left medial sympathetic venous line can be recognized in the albino rat. The venous line *A* which develops in the embryos of group 5 (15 mm. C.R. length) does not correspond with any of the previously described venous lines. It is not necessary to give this a specific name and the left azygos vein caudal to intercostal vein 5 and the inferior hemi-azygos vein may be described as developing from the perisympathetic plexuses. The main feature of the perisympathetic plexus portion of these veins is that it lies medial to the sympathetic trunks. Their relation to the segmental arteries in the albino rat differs from that in man in that occasional segments of the left azygos vein pass dorsal to the intercostal arteries (Greene, 1935). The inferior hemi-azygos vein in the rat lies dorsal to the intercostal arteries whereas in man it lies ventral.

In the rabbit the intercostal veins drain temporarily into the thoraco-lumbar venous line since the postcardinal veins disappear before the medial sympathetic venous lines appear (Reagan & Tribe, 1927). The thoraco-lumbar venous line is derived from the perisympathetic plexus and has no adult derivative. It was not found in the albino rat. Auer (1946) failed to find this vein in a series of human embryos.

In man the azygos veins anastomose caudally with the subcardinal portion of the inferior vena cava (Seib, 1934). These connexions are developed from the perisympathetic plexuses and are not homologous with the temporary connexions between the lowest intercostal veins and the subcardinal veins found in embryos of group 3.

No attempt is made to give the venous line *A* a specific name; it is regarded as a part of the perisympathetic plexus. The extreme variability of the azygos venous

system, with the exception of the parts developed from the anterior portion of the postcardinal, is due to its development from a plexus of veins. Further attempts to analyse this plexus will only add to an already confusing terminology. Auer (1946) suggests the retention of the term supracardinal vein for that part of the perisympathetic plexus which lies medial to the sympathetic trunks. Even this is unnecessary if this venous plexus be regarded as consisting of a perisympathetic and a retro-aortic portion.

CONCLUSIONS

1. The major part of the left azygos vein, the inferior hemi-azygos vein and the retro-aortic portions of the 6th–10th right intercostal veins are developed from the perisympathetic plexuses.

2. The left postcardinal vein forms the left azygos vein from its junction with the left superior vena cava to the level of the 5th or 6th intercostal vein.

3. It is not necessary to give a special name to each longitudinal venous line developed from the perisympathetic venous plexus.

I wish to thank Prof. H. A. Harris for advice and encouragement, Dr F. P. Reagan for advice on the technique of injecting embryos, and Messrs J. W. Cash and E. A. King for technical assistance.

ABBREVIATIONS

A.	Aorta	*N.*	Notochord
D.B.A.	Dorsal basal anastomosis	*Q.L.*	Quadratus lumborum
C.A.	Capillary arch	*R.A.V.*	Right anterior cardinal vein
D.A.R.	Dorsal aortic ramus	*R.C.*	Ramus communicans
I.N.	Intercostal nerve	*R.V.A.*	Right venous line *A*
L.A.V.	Left anterior cardinal vein	*R.P.V.*	Right postcardinal venous line
L.V.A.	Left venous line *A*	*R.S.V.*	Right subcardinal venous line
L.M.S.	Left medial sympathetic venous line	*R.Sc.V.*	Right subcentral venous line
L.P.V.	Left postcardinal venous line	*S.*	Sympathetic cord
L.S.V.	Left subcardinal venous line	*S.V.*	Segmental vein
L.Sc.V.	Left subcentral venous line		

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THE PRONEPHROS AND THE EARLY DEVELOPMENT OF THE MESONEPHROS IN THE DUCK

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INTRODUCTION

The external glomeruli of the avian embryo were described by Gasser (1879), Balfour & Sedgwick (1878, 1879) and Sedgwick (1880, 1881). Sedgwick described the detailed development of the external glomeruli in the chick and showed that they were continuous caudally with the Malpighian bodies of the mesonephros through a short transition zone. At this level, external and internal glomeruli were seen to be continuous with each other through their vascular cores. A similar series of external and transitional glomeruli was described by Wiedersheim (1890) in the Crocodilia and Chelonia, and was considered by him to represent a gradual cranio-caudal transition from the pronephros to the mesonephros. The external glomeruli of the duck were stated by several authors (Sedgwick, 1880; Mihalkovics, 1885) to be better developed than in the chick. Accordingly, the early excretory system of the duck has been compared with that of the chick, crocodile, alligator and turtle.

MATERIAL

Duck embryos of the following incubation ages were used: 32, 56, 64, 88 and 96 hr., $4\frac{1}{2}$, $5\frac{1}{4}$, $6\frac{1}{4}$, $6\frac{3}{4}$, 7, 8, 9, 10, 12 and 15 days. They were fixed in Bouin's solution and cut in serial sections at 5μ, and stained with haematoxylin and eosin. Those in the coronal and sagittal planes were found to be of great value.

A large number of chick embryos from the collection of the Cambridge Anatomy Department was also studied, as well as a number of crocodile, alligator and turtle embryos. The chicks ranged from about 36 hr. to 5 days. The crocodile embryos measured 13, 14, 16 and about 40 mm., the alligator embryos 18, 20 and 25 mm., and the turtle embryos 4·5 and 7·5 mm.

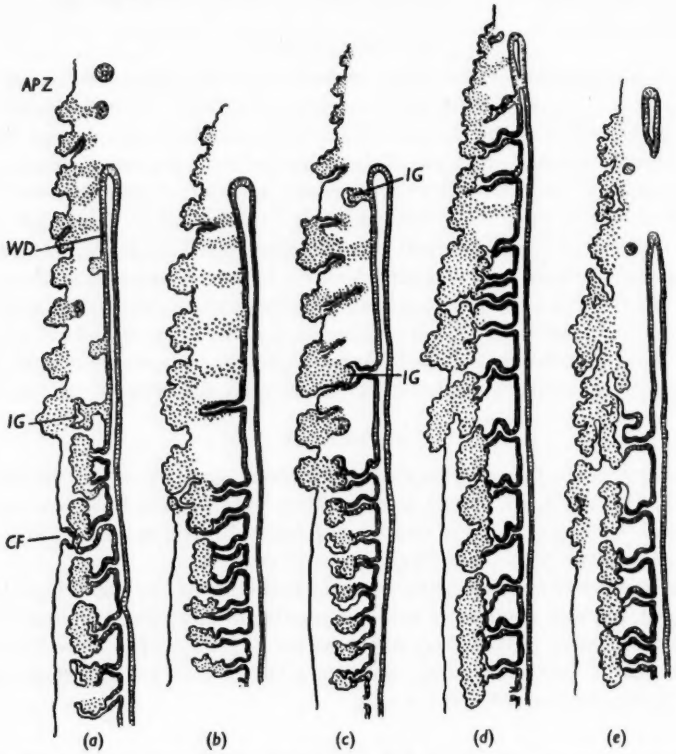
NOTE ON TERMINOLOGY

A glomerulus is considered to be 'external' when it hangs freely into the peritoneal cavity. It is considered as 'internal' when it lies within the Wolffian ridge enclosed within a capsule. Such an internal glomerulus with its capsule constitutes a Malpighian body.

RESULTS

The segmentation and differentiation of the intermediate cell mass in the duck embryo is essentially the same as in the chick. The papers of Sedgwick (1881) may be consulted for a complete and detailed account of the slight differences between them. The segmentation process which divides the paraxial mesoderm into somites fails to extend into the region of the intermediate cell mass, which remains, therefore, as a more or less unbroken column of cells adherent to the coelomic epithelium. The nephric tubules later arise in this tissue as cords or masses of cells which are not

segmental and which retain for a considerable time their contact with the coelomic lining. These areas of contact are particularly clear in those segments in which the external glomeruli are later developed. Caudally, and at a later stage, the intermediate cell mass loses its connexion with the coelomic epithelium and forms a longitudinal nephrogenic cord in which the mesonephric tubules arise.



Text-fig. 1. Diagrammatic reconstruction of the forepart of the primitive excretory system in duck embryos of (*a*) 96 hr., (*b*) and (*c*) 88 hr., right and left sides, (*d*) 4½ days, and (*e*) 5½ days. The left side (*c*) of the 88 hr. embryo is shown in reverse. The diagrams are not drawn to scale.

In an embryo of 64 hr. three fairly well-defined zones may be seen. From the 6th to the 10th somite there is a zone in which no trace of the Wolffian duct can be found; there is, however, a number of large nephrogenic masses closely connected to the coelomic lining. These are seen in the same area in an embryo of 72 hr., and occasionally they contain a minute slit-like prolongation of the body cavity, a rudimentary nephrostome. This zone probably corresponds with the abortive area from the 7th to the 11th somite described by Sedgwick (1880) in the chick. The primordium of the Wolffian duct was identified from the 10th to the 15th somite in embryos of 56 and 64 hr., apparently differentiating at many points from the intermediate cell mass through which it was continuous with the coelomic epithelium.

Caudal to the 15th somite there was a continuous nephrogenic cord connected with the coelomic epithelium at many points but containing no tubule primordia.

The condition of the nephric system in an embryo of 96 hr. is illustrated diagrammatically in Text-fig. 1*a*. Anterior to the dilated upper end of the Wolffian duct there is an abortive zone containing two well-differentiated vesicles, the more caudal of which is connected to the thickened coelomic epithelium by a cluster of cells. Caudal to this there is a clump of cells also connected to the coelomic lining which is here invaginated to form a rudimentary nephrostome. There is a primordium of an external glomerulus opposite this clump of cells and the more caudal of the two vesicles. Caudal to these there is a series of six coelomic thickenings, or primordia of external glomeruli, making eight in all in this embryo. The relations of the nephrogenic tissue to these glomeruli are shown in the same diagram. In some cases the glomerulus is connected to the Wolffian duct by a cord of cells, indicated by stippling; there are two such cords opposite the third glomerulus. In other cases there is a mass of nephrogenic cells unconnected as yet with the duct, or there may be an actual vesicle. A third variation is a clump of cells or a vesicle adherent to the wall of the Wolffian duct but not to the peritoneal surface. These variations are dependent on the degree of separation of the intermediate cell mass from the coelomic epithelium and from the duct primordium. Ultimately the majority of these cell clusters or vesicles will form more or less well-differentiated tubules, usually with their own internal glomerulus. The last of the group of external glomeruli in the same diagram is already fused with an internal glomerulus forming the first of a series belonging to the mesonephros proper. Thus the transition zone, as this fused external and internal glomerulus may conveniently be termed, is short at this stage.

An embryo of 88 hr. is illustrated in Text-fig. 1*b*, *c*. This embryo was actually somewhat further advanced than the 96 hr. specimen, as judged by its general development. The degree of development on opposite sides of a single embryo is usually very different and must be emphasized. There is in this specimen a tendency towards increase in size and fusion of the external glomeruli. Their number is approximately the same as in the previous embryo. The cellular cords are present as before except that one of them has become a well-differentiated tubule opening into the Wolffian duct (Text-fig. 1*b*). On the opposite side two cords, one near the upper end of the duct, possess a clearly defined vascular glomerulus which is truly internal and, with its capsule, forms a typical Malpighian body (Text-fig. 1*c*). A transition zone no longer exists since there are at least three internal glomeruli, one of which is almost opposite the cranial end of the Wolffian duct. The duct is always dilated at its cranial end as described by Gasser (1877) and Sedgwick (1880) for the chick. Rudimentary nephrostomes are present here and there, but rarely if ever communicate with the tubules or their primordia.

In an embryo of 4½ days the external glomeruli are conspicuous structures (Text-fig. 1*d*). They consist not of a series of discrete bodies as in the early stages but form a continuous mass of vascular glomerular tissue hanging into the body cavity on the surface of the Wolffian body. Another important feature at this stage is the presence, deep to the external glomeruli, of at least eight well-differentiated internal glomeruli enclosed within a Bowman's capsule and forming true Malpighian bodies. There are two or three others still represented by cellular cords, and it is not

clear whether they too will become tubules and Malpighian bodies or will degenerate. Sedgwick also observed the cellular cords connecting the external glomeruli with the Wolffian duct in the chick but was unable to determine whether they became tubules or not. In the duck the majority of them certainly do, but in the chick they are rudimentary and soon disappear. There is, however, great individual variation.

The external glomeruli and the associated nephric elements at about 4½ days have now reached their maximum development. Later the glomeruli undergo further fusion and progressive fibrosis and shrinkage. The appearance and relationships of the excretory system at this stage are shown in Pl. 2, fig. 5. It bulges into the pleuro-peritoneal canal, the walls of which are thickened so that the glomerulus with its contained cavity superficially resembles a typical Malpighian body. The external glomeruli are always in relationship to the dorsal wall of the pericardial cavity until this becomes closed off (see Lillie, 1919). The rapid fibrosis which affects the external glomeruli, particularly at the cranial end, also involves the Malpighian bodies deep to them. At all stages after the fifth day the latter appear almost if not entirely co-extensive with the external glomeruli. The zone of fused external and internal glomeruli is shown in Text-fig. 1*e* from an embryo of 5¼ days in which, however, the associated tubules were largely fragmented and fibrosed. As a rule at least five of the internal glomeruli with their tubules persist in this zone till as late as the 7th or 8th day.

The histogenesis of the external glomerulus is illustrated in Pl. 1, figs. 1–4. It begins as a thickening of the coelomic epithelium which bulges into the dorsal wall of the body cavity. It is a very compact structure at first, but becomes excavated by blood vessels which appear to be formed *in situ* before any connexions with the aorta are established (Sedgwick). The glomeruli do not at any time show a segmental arrangement and new ones are formed at later stages between the original glomeruli; in this way the originally discrete bodies become converted into a continuous structure. Afferent and efferent connexions are soon established with the aorta and cardinal vein respectively. The glomerulus is characteristically sac-like and packed with blood cells at this stage (Pl. 1, figs. 1, 2). The short cord of cells which has been described uniting the external glomerulus to the Wolffian duct also shows cavitation and the formation of blood vessels (Pl. 1, fig. 1). Those cavities which do not become lined with endothelium to constitute glomerular capillaries become the cavity of Bowman's capsule and the lumen of the tubule, which quickly opens into the Wolffian duct (Pl. 1, fig. 2). These Malpighian bodies formed in relation to the external glomeruli are tardy in their development compared with those of the mesonephros proper. They are rudimentary or absent in most of this region in the chick. Since they arise from the same parent tissue as the external glomeruli, the two structures necessarily show an intimate relation to each other (Pl. 1, fig. 3). This continuity is emphasized by the fusion of their vascular cores and both are supplied by the same afferent arteriole (Pl. 2, fig. 6). The angioblastic activity of the coelomic epithelium becomes extended to involve the nephrogenic tissue and later the stroma of the urogenital ridge. Thus in places a distinction between the external and internal glomeruli becomes impossible (Pl. 1, fig. 4). Here there is a mass of angioblastic glomerular tissue bulging slightly into the body cavity and also extending deeply towards the neck of a nephric tubule. Another feature of the excretory system in the

region of the external glomeruli is the tendency for the walls of the cavities within the glomerular tissue to become attenuated and finally to break down. This results in a loosening of the texture of the glomeruli and also in an extension of the glomerular capsule. Thus in Pl. 1, fig. 4, the capsular cavity and the peritoneal cavity have become confluent by breakdown of the lateral attachments of the glomeruli to the urogenital ridge. This is not an artefact and is of frequent occurrence. The communication so established between the tubule and the peritoneal cavity is probably not the remains of a nephrostome as illustrated in Pl. 1, fig. 4. Nephrostomes are always rudimentary in the duck, as in the chick.

Mention must be made of the development of the Malpighian bodies caudal to the external glomeruli. They are of interest from their unusually large size both in the duck and chick (Text-fig. 1 and Pl. 2, fig. 7). The nephrogenic tissue from which they are derived at an early stage (e.g. 88 hr.) is voluminous and clumped into elongated masses extending over many sections. These masses are developed when the angioblastic activity of the mesoderm is intense in all parts of the embryo. The formation of blood vessels and the growth of the capsule by cavitation proceed *pari passu*. The Malpighian bodies thus formed (about nine in number) are large and elongated, have each up to three tubules connected with them, are provided with multiple efferent vessels and contain a very complicated capsular cavity. These bodies also show fusion and breakdown with resulting confluence of their cavities (Pl. 2, fig. 7). They become progressively smaller and simpler in a cranio-caudal direction, gradually merging with the small discrete Malpighian bodies posteriorly.

EXTERNAL GLOMERULI OF THE CHICK

The number of coelomic proliferations representing primordia of the external glomeruli in the chick are much more variable in number and degree of development, and in the majority of 3-day embryos studied only two or three primordia could be identified. The significant difference between the chick and the duck is that in the former the Wolffian duct is either not present in the region of the external glomeruli or is merely represented by a series of vesicles. In the duck the duct is well developed and patent throughout the entire glomerular area except in the later stages when the latter is degenerating. Internal glomeruli are formed only in relation to the caudal two or three external glomeruli. In most of the embryos studied there was no 'transition zone' at all, the first internal glomerulus lying entirely caudal to the external glomeruli. Only the caudal two or three of the external glomeruli attain a significant size in the chick. The excretory system throughout the external glomerular region has all the characters of a vestigial organ.

EXTERNAL GLOMERULI OF THE REPTILE

No very young stages were available, but all the specimens examined show a well-developed external glomerulus comparable in all its features with that of the duck (compare Pl. 2, figs. 5, 6). The only significant differences seem to be the greater development of the associated tubules and the presence of ciliated nephrostomes, though the latter are by no means constant. Internal glomeruli are found to be almost co-extensive with the external glomeruli, but are larger and more irregular

in shape than they are in the duck. The extreme irregularity of these glomeruli, associated with breakdown of glomerular septa and the production of a complex capsular space, is shown in coronal section (Pl. 2, fig. 9).

DISCUSSION

The S-shaped cords of cells which unite the primordium of the Wolffian duct to the coelomic epithelium in early embryos are familiar to all students of avian embryology (Kölliker, 1879; Sedgwick, 1880). They are the rudiments of the nephric tubules and were homologized by Sedgwick with the segmental tubules of the Elasmobranchii. They often contain a slit-like extension of the coelomic cavity, a vestigial nephrostome. These cords, which remain after the intermediate cell mass has separated from the somite in each segment, are very variable in number and extent. The following account is based on Sedgwick's description. They are found from the 7th to the 20th segment in the chick, but in the duck are found not only in this area but more caudally throughout most of the segmented part of the embryo. Two or three are found in each segment. In the chick an external glomerulus is developed opposite the cords from the 11th to the 13th somite in an embryo of thirty somites, the extent of their development being very variable (maximum limits from the 9th to the 13th somite). Only the caudal two or three external glomeruli become well developed, the rest soon disappearing. The caudal two or three become fused deeply with true Malpighian bodies of the mesonephros, the internal and external glomerular formations being thus continuous by their vascular cores and sharing the same afferent artery. The tubules associated with them remain rudimentary but open into the Wolffian duct. The latter is fragmented and of uneven calibre above this point.

According to Sedgwick, the nephrostomes and the canals to which they lead in each of the S-shaped cords widen out in the area of fusion and become a recess or bay of the peritoneal cavity bounded on each side by a fold. The bay is prolonged caudally for a short distance and appears in transverse section as a closed cavity which however opens out into the peritoneal cavity in more cranial sections. An external glomerulus is developed in relation to the open (cranial) part of the coelomic bay; an internal glomerulus is formed in relation to the closed (caudal) part of the bay. The two glomerular formations are continuous and the external glomerulus is regarded merely as an internal glomerulus bulging outwards through the open mouth of the coelomic bay into the body cavity. The caudal part of the bay, with its enclosed internal glomerulus, forms a typical Malpighian body of the mesonephros.

An essentially similar process is described in the Crocodilia and Chelonia by Wiedersheim (1890) and in the Chelonia by de Walsche (1929). According to de Walsche the tubules of the pronephros (6th to 9th segments in the Chelonia) open directly into the coelomic cavity by a ciliated nephrostome. The opening of the pronephric tubule later widens, and its medial wall becomes moulded on to the rapidly enlarging external glomerulus. In the transition zone the coelomic opening becomes progressively narrowed and finally closed. In this way the ciliated nephrostome becomes cut off inside a 'pronephric chamber' and no longer opens directly into the coelomic cavity. The external glomerulus similarly becomes divided by closure of the coelomic bay so that part of it is cut off in relation to the pronephric chamber and part

remains external, hanging freely into the body cavity. The enclosed part of the external glomerulus and the pronephric chamber now form a typical Malpighian body of the mesonephros. Wiedersheim's account of the process in the Crocodilia is very difficult to follow but is essentially the same.

The manner of fusion of external and internal glomeruli in the duck, described in this paper, is different in several important particulars from that observed by Sedgwick, Wiedersheim and de Walsche. The area over which fusion occurs is more extensive in the duck than in the chick and allows a more accurate study of the conditions present. At least nine well-developed external glomeruli fused with internal glomeruli are to be seen in the duck embryo of $4\frac{1}{2}$ days. Rudimentary nephrostomes are found at an earlier stage (Pl. 1, fig. 1), but are uncommon and transient. No coelomic bay could be identified as described by Sedgwick and no process of encapsulation of an external glomerulus by peritoneal folds was observed at any stage. The cavity of Bowman's capsule in the Malpighian bodies of this area developed quite independently of the coelomic cavity (Pl. 1, fig. 2) and never in continuity with a coelomic bay. Continuity between the capsular space and the peritoneal cavity was developed occasionally by attenuation and breakdown of the glomerular septa (Pl. 1, fig. 4). This breakdown of septa by continued extension of cavitation and 'loosening up' is a typical feature of glomerular tissue and leads, in the cranial part of the mesonephros, to the confluence of adjacent capsular spaces and to the formation of a complex and often ragged cavity (Pl. 2, figs. 8, 9).

The fundamental developmental feature of the pronephric and transition zones of the primitive excretory organ in the bird seems to be the intense angioblastic activity of the coelomic epithelium at an early stage. Further extension of this activity leads to fusion of the external glomeruli into a continuous 'glomus' and also involves the Wolffian stroma deeply. Where Malpighian bodies are in process of development the same angioblastic activity involves them also so that the external glomeruli appear to be growing into the primordia of the Malpighian bodies. The immaturity of a Malpighian body at this stage as compared with the external glomeruli is illustrated in Pl. 1, fig. 2. It is often impossible to differentiate the external glomerulus from the internal glomerulus, the two forming a fused mass of glomerular tissue (Pl. 2, fig. 9). In the crocodile and turtle the fusion is even more intimate and the internal glomeruli are not rounded but have a somewhat strand-like pattern (Pl. 2, fig. 9).

Three parts, then, may be observed in the early excretory organ of the bird and some reptiles (crocodile and turtle):

(1) A region, anterior to the first true Malpighian body of the mesonephros, which contains external glomeruli, nephrostomes and tubules. It may justifiably be termed 'pronephric' on these grounds. It is rudimentary in the chick and the duck. In the crocodile and turtle it contains well-differentiated tubules and ciliated nephrostomes as well as external glomeruli. It is probably a short zone even in these reptiles (three to four tubules, according to de Walsche).

(2) An intermediate zone, in which fused external and internal glomeruli are found. It is well developed in the crocodile and turtle and, with the pronephros, involves about fifteen tubules (Wiedersheim). It is mainly rudimentary in the chick. In the duck it is an extensive and probably functional zone, though retarded in its development as compared with the rest of the mesonephros.

(3) A caudal zone, which contains no external glomeruli and which belongs to the mesonephros proper.

SUMMARY

1. The early development of the excretory system in the duck has been studied. A series of well-developed external glomeruli are formed from the coelomic epithelium. Later, internal glomeruli are formed in the same area and become fused with the external glomeruli.

2. Sedgwick's account of the development of this part of the avian excretory system is not confirmed.

3. The early excretory system of the crocodile and turtle is compared with that of the duck and shows striking resemblances. The duck appears in this respect more primitive and reptilian than the chick.

Grateful acknowledgement is made to Prof. H. A. Harris and Prof. D. V. Davies for their advice, to Mr J. A. F. Fozzard for the photographs and to Mr J. W. Cash for technical assistance.

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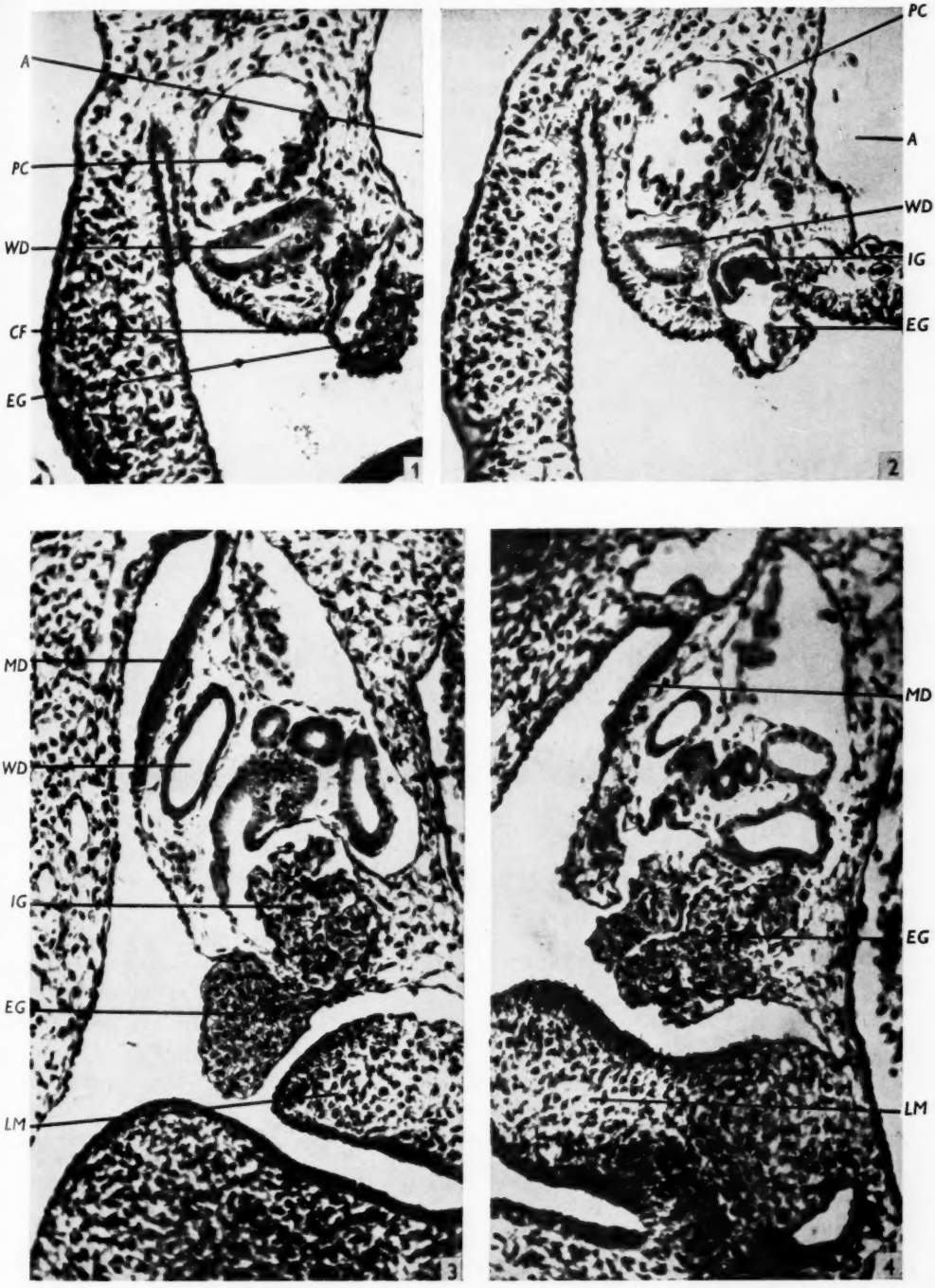
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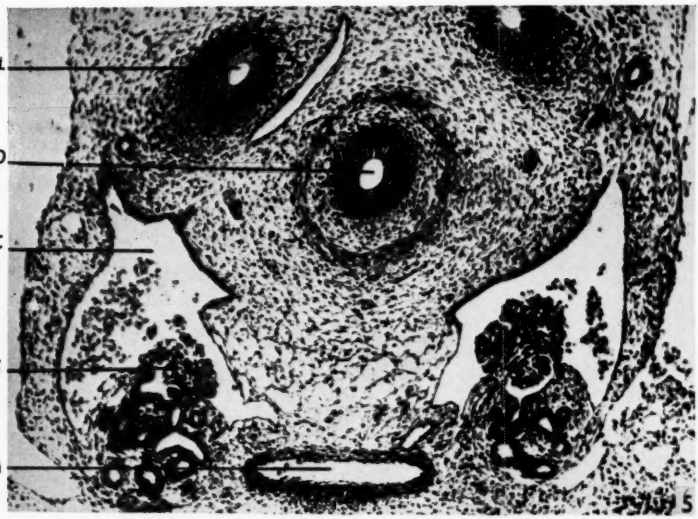
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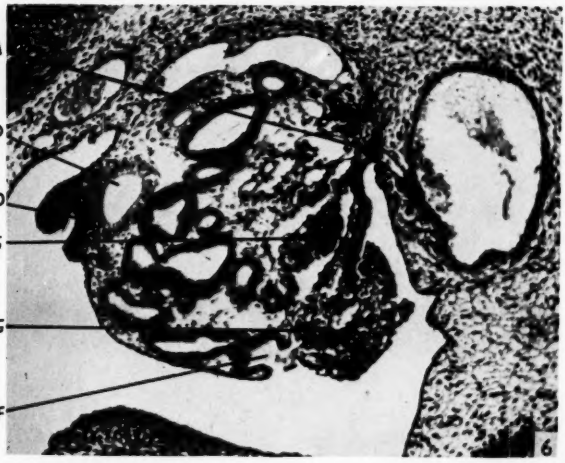
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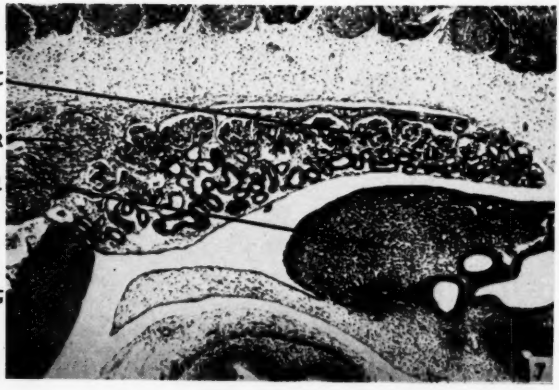
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ABBREVIATIONS

A	Aorta	*L*	Lung
AA	Afferent artery	*LM*	Lateral mesocardia (Lillie)
APZ	Abortive pronephric zone	*MD*	Müllerian duct
CC	Capsular cavity	*O*	Oesophagus
CF	Coelomic funnel	*PC*	Posterior cardinal vein
EG	External glomerulus	*PPC*	Pleuro-peritoneal canal
G	Gonad	*SR*	Suprarenal
GC	Glomerular column	*WD*	Wolffian duct
IG	Internal glomerulus		

EXPLANATION OF PLATES

Plate 1

Fig. 1. Transverse section of duck embryo of 88 hr. to show the developing external glomerulus. There is a rudimentary nephrostome. ×245.

Fig. 2. Transverse section of duck embryo of 88 hr. showing the developing external and internal glomeruli. Note how the vessels of the external glomerulus appear to be growing into the primordium of the internal glomerulus. ×245.

Fig. 3. Transverse section of duck embryo of 4½ days through the caudal third of the external glomerular area. There is a well-developed and very vascular internal and external glomerulus. The tubule is well differentiated. ×245.

Fig. 4. Transverse section through the caudal third of the external glomerular area in the same embryo as fig. 3. The internal and external glomerulus are not clearly demarcated from each other. The capsular space of the former has broken through to the general body cavity. ×245.

Plate 2

Fig. 5. Transverse section of a duck embryo of 5¼ days to show the external glomeruli and associated parts lying in the dorsal wall of the pleuro-peritoneal canal. Note the thickening of the coelomic epithelium in the ventral wall of the latter. ×48.

Fig. 6. Transverse section of a crocodile embryo. Note the external and internal glomeruli and the afferent artery to both. The nephrostome is ciliated, the tubules well differentiated. Note the developing Müllerian duct. ×98.

Fig. 7. Sagittal section of the mesonephros of a duck embryo of 7 days showing the massive and fused glomeruli in its cranial third. Note the relation of the gonad to the mesonephros. ×45.

Fig. 8. Sagittal section of a chick embryo of 5 days through the cranial part of the mesonephros. There is extensive fusion of the glomeruli and breakdown of adjoining capsular walls. ×98.

Fig. 9. Coronal section of a crocodile embryo showing the fused external glomeruli and the underlying glomeruli which are irregular and in the form of fenestrated plates. ×45.

THE STAINING OF NERVE FIBRES IN PARAFFIN SECTIONS WITH SILVER*

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INTRODUCTION

The primary requirement for all forms of histological research is a series of methods which will consistently demonstrate particular features of a tissue and, wherever possible, indicate the chemical composition of the structure which is stained.

The multitude of existing methods for staining nervous elements with silver is a good measure of the relatively unsatisfactory nature of all, and demonstrates that none fulfils all the criteria necessary for a perfect staining method.

The criteria which were kept in mind during this research were:

(1) That the stain should be specific for nervous elements or, if other structures were stained, they should be differentiated easily by means of colour contrast.

(2) That the stain should demonstrate every nervous element (excluding neuroglia) on all occasions with equal certainty, that it should be simple to prepare and independent of any complex substances of unknown chemical constitution.

(3) That it should be possible to use such a stain with paraffin sections, thus avoiding the irregular staining and brittle nature of pieces of tissue immersed in a silver solution, and the lack of cohesion between the various parts of frozen sections, while maintaining the advantages of thin serial sections, alternate members of which may be stained by different methods.

(4) That the stain should function with tissue from a number of standard fixatives, and should not require fixation with a solution which precludes the use of other staining methods.

The earlier silver methods, mainly based on Cajal or Bielschowsky techniques, though excellent for many purposes, all depend on bulk staining or the use of frozen sections and many require very special fixatives. Of these methods few are completely reliable even in the hands of expert technicians and much valuable experimental material may be lost at the last ditch.

More recently, a series of techniques for staining paraffin sections has been evolved and some of these are extremely valuable, notably Bodian's (1936) method. All of them have, however, certain drawbacks which are not immediately obvious.

Bodian's method makes use of protargol, a strong silver proteinate, now manufactured only by the Winthrop Chemical Company in the United States. This protargol is the only member of a large series of strong silver proteinates which stains nervous tissue adequately, but nothing is known of its composition beyond the fact that it is a combination of silver with partially hydrolysed egg albumen. Even protargol is not uniformly satisfactory, for only certain samples produce the desired result while others (Holmes, 1943), even those manufactured by the same Company (Green, 1947*a*), are inactive.

* Accepted by Edinburgh University for the Gunning Victoria Jubilee Prize in Anatomy, 1948.

Silver's (1942*a*) method is a modification of one of the standard techniques for silvering glass in the production of mirrors. It makes use of a mixture of Rochelle salt, silver nitrate and potassium sulphide added to an equal volume of ½ % protargol, but has the disadvantage that it is complex to prepare, capricious in action and has all the disadvantages of Bodian's technique.

Holmes (1943) gives an excellent review of silver techniques for staining paraffin sections, and notes that silver oxide and silver carbonate are more effective for this purpose than silver nitrate. He was apparently unaware of the presence of silver chloride in protargol and gives no indication of having used this salt. Holmes makes use of a very weak solution of silver nitrate (1:10,000–1:100,000) with a borax-boric acid buffer. Despite its simplicity this method has not given adequate results in my hands, though the reason for this is not at all obvious. In any case it requires variations in pH and silver concentration for different tissues, which is unsatisfactory where sections passing through a whole animal or embryo are to be stained.

Pearson & O'Neill (1946) make use of a form of physical development. Sections are first impregnated with silver nitrate and the deposit intensified by means of a solution containing silver nitrate and a reducing substance (hydroquinone) with a protective colloid (gelatin) added to arrest excessive deposition of silver. The method is well conceived but leads to the deposition of silver around the axons making them appear thicker than in fact they are, as is the case with many of the block impregnation methods. Also it is not a highly specific stain and for this reason works best with embryonic tissues where few connective tissue fibres are present to complicate the picture. Pearson (1947) has stated that the preliminary use of protargol in addition to the silver nitrate is valuable. Gelatin, too, is a notoriously variable substance and results may depend on the type used, the presence or absence of traces of mustard oil or sodium chloride perhaps playing an important part in the results obtained.

In a previous publication (Romanes, 1946) a method was described giving results similar to those obtained with Bodian's technique. The solution consists of colloidal silver produced by the action of tannic acid on a weak (0·1 %) ammoniacal silver nitrate solution and made alkaline by the addition of pyridine. This method, though reliable, depends on two variables which are difficult to control. As with all ammoniacal silver nitrate solutions it is difficult to determine the exact end-point when all the silver hydroxide, precipitated by the ammonia, has just been redissolved and, as stated above, variations in the type of gelatin used and the pH of the final solution has a profound effect on the impregnation obtained.

EXPERIMENTS

A. *Protargol and other silver proteinates*

The present research was aimed at removing some of these variables.

Since it had been found that Bodian's method gave the best result an attempt was made to analyse the differences between protargol and the other inactive silver proteinates, on the basis of information available in the chemical literature. The significant points may be briefly summarized:

(1) Protargol is paler in colour than any other silver proteinate, with the possible exception of freshly prepared silver gelatose (Martindale, 1941).This suggests that

the particle size of the silver in protargol is smaller than that in other silver proteinates.

(2) Protargol alone gives the tryptophane reaction (Mannich & Gollasch, 1927).

(3) Protargol, while containing about the same total amount of silver (7·91 %) as the other proteinates, has a percentage (0·83 %) of silver as chloride (Kolthoff & Tomicek, 1925).

From these facts it seems possible that the success of protargol may be due either to the small size of the silver particles, or to the nature of the protein hydrolysate used in its manufacture, or to the presence of silver chloride.

Tests made of the pH of 1 % solutions of various silver proteinates, including protargol, show that the majority have a pH of about 7·8–8. Thus the pH alone cannot be responsible for the failure of some solutions and the success of others.

The first attempts were directed to the production of a silver proteinate with characters similar to that of protargol.

Various hydrolysates of albumen, casein and gelatin were prepared by the action of either trypsin or papain or alkalis, and the results of hydrolysis were either precipitated by silver nitrate or had small quantities of silver nitrate added to them.

In the former the precipitate was washed and brought into solution by the addition of sufficient alkali to raise the pH to 7·6–7·8. In the case of the casein hydrolysates the precipitate was found to redissolve following repeated washing with distilled water. The pH was then between 7·5 and 7·8. Though a small amount dissolved in each wash it was only when this point was reached that the greater part passed into solution.

These solutions ($\pm$ metallic copper) were used in lieu of protargol for impregnating sections, which were subsequently developed and gold-toned by the method previously indicated (Romanes, 1946). Many of the possible variables were checked by altering the concentration, the pH, the type of alkali added, the fixation of the tissue and the time and temperature of the impregnation.

Despite occasional successes the results were poor and difficult to control, which is not surprising in view of the complicated nature of the solutions used. Certain points, however, did emerge:

(1) The results obtained when the solution was made alkaline either with ammonia, ethylamine or pyridine, were definitely superior to those obtained with sodium or potassium hydroxide.

(2) Casein or egg albumen hydrolysates gave good results which could not be obtained with gelatin.

(3) The pH range for successful results lay above 6·8 and below 8, with the optimum about 7·8. Below 6·8–7 only a greenish, granular staining of all the tissues resulted, while above 8 all specificity was lost.

(4) Impregnation in the cold seldom gave good results, while at 58° C. in the dark much better impregnation was obtained.

(5) Successfully impregnated sections were light brown in colour before development and darkened only slightly in the developer suggesting, as Holmes (1943) found, that the important part of the process consists of a reduction of silver by the tissue itself (vide infra).

Finally, to test the value of silver chloride, small quantities of sodium chloride, commensurate with the amount found in protargol, were added to the silver

precipitate of casein hydrolysate. This addition was found to raise the pH and bring the precipitate partially into solution. This procedure seemed to improve the staining but dissolved the albumen fixing the sections to the slides so that few satisfactory results could be obtained.

B. *Silver chloride*

(*a*) *General experiments*

In view of the failure with these tests it was decided to investigate the results with silver chloride.

Initially, a silver chloride suspension was produced by adding 30 c.c. of 0·1 % silver nitrate to 100 c.c. of 0·1 % gelatin followed by an equivalent amount of 0·1 % sodium chloride. This was brought to pH 7·8 with a small quantity of very weak ammonia solution and sections were stained for varying periods at 58° C. in the dark. The subsequent phases of developing and gold toning were carried out as before except that 2 % oxalic acid without formalin was used to reduce the gold chloride. It was found, as expected, that during impregnation the pH gradually fell, presumably on account of the evaporation of the ammonia, and overnight was reduced to about 7. Much of the silver chloride precipitated out in the first 2 hr. of impregnation, but despite this the stain was good and most of the precipitate on the slides was dissolved by the sodium sulphite in the developer.

In order to maintain the silver chloride in suspension the amount of gelatin was increased, but this was found to impair the staining more or less in proportion to the increase in the amount of gelatin added.

It was therefore decided to reduce the concentration of silver chloride in the solution and dispense with the gelatin which was an unsatisfactory constituent. As a result of this it was found that stable silver chloride suspensions in distilled water were obtained with solutions containing not more than 0·01 % of silver nitrate and the equivalent amount of sodium chloride. Solutions of this type gave results superior to those obtained with the addition of gelatin.

The next process was to vary each constituent of this simple solution in turn and decide the optimum solution for staining purposes, then to try the effect of adding other substances to the stain and the use of other silver salts.

The concentration of silver nitrate was gradually reduced by a series of steps to 0·0003 % with the equivalent quantity of sodium chloride added in each case. This produced very little change, and though it was obvious that the staining at the lowest concentration was slightly less intense than that at the highest concentration, it was practically impossible to differentiate between sections stained in solutions containing 0·003 and 0·0003 % of silver nitrate respectively. A standard solution of 0·003 % silver nitrate was therefore chosen for further tests.

(*b*) *Variations of* pH, *type of fixative, sodium chloride concentration, and impregnation time*

As has been pointed out above, the pH falls steadily but slowly during impregnation at 58° C. When this fall in pH was allowed to proceed to about 6·5–6·9 it was found that the final sections had a greenish colour and showed only a granular, non-

specific deposition of silver. The actual point at which this change occurred varied with the fixative. Sections of material fixed in formalin showed no loss of specificity even if the pH dropped to 6·8, but if chloral hydrate (10 %) was added to the formalin this greenish, granular appearance became obvious if the pH dropped below 7.

A similar result was obtained with Bouin-fixed material and where metallic copper (vide infra) was added to the impregnating fluid. With these fixatives and with copper it was therefore found necessary to begin impregnation at a higher pH (8) when a standard time of 16 hr. was given for impregnation.

It is interesting in this connexion to note that in the previous publication (Romanes, 1946) it was observed that over-impregnation led to a gradual diminution in the staining of the tissue and, though not indicated at that time, finally to a greenish granular deposit. Recent tests of the pH of the solution then used at different times during impregnation show that over-impregnation is associated with a reduction of the pH below 7.

At first pH readings were made with a glass electrode but it was found that sufficient accuracy could be obtained with comparator papers (Messrs Johnston and Co.). As this was a distinct asset for most histological laboratories, all figures given here have been estimated by this means, although the readings are considerably lower than with the glass electrode.

The question arose whether exactly equivalent amounts of sodium chloride and silver nitrate were essential or not. To test this the concentration of sodium chloride was varied within wide limits. It was found that as the concentration of sodium chloride was reduced below the equivalent amount the stain became denser and progressively less specific, giving results similar to those obtained when silver nitrate (0·003 %) was used alone. On the other hand, when the sodium chloride exceeded the equivalent amount, no specific staining could be obtained and the results were similar to those which followed when the pH of the standard solution was allowed to fall too low. In order to avoid this difficulty it was decided to keep the silver nitrate solution very slightly higher (3:1) than the equivalent amount (2·9:1) with slight loss of specificity but avoiding the above result which could easily supervene from slight errors in measurement.

An attempt was made to standardize the impregnation time. It soon became obvious that it was not in a simple inverse relation to the temperature, but varied with the type of tissue used and to a less extent with the nature of the fixative. It was also noted that, provided the pH was not allowed to fall too low, long periods of impregnation produced no appreciable difference. For this reason and because those tissues which required the longest impregnation (cerebral cortex) had reached their maximum degree of staining in less than 16 hr. at 58° C. this was taken as the standard time.

(*c*) *Other silver salts*

At this stage it became necessary to determine whether or not silver chloride was essential for the success of this staining method, for, if it was, it might explain why certain batches of gelatin (some of which contained sodium chloride) proved successful in the previous stain (Romanes, 1946) while others did not.

To determine this, certain other silver salts were used in the same way, being made alkaline with ammonia:

(1) Silver nitrate. When this salt was used alone even in high dilution the staining was diffuse and non-specific for nervous tissue, despite considerable variation in the pH range.

(2) Silver bromide and iodide. These two halides were both tested extensively because of their similarity in physical properties to silver chloride. All three are light-sensitive, but both the bromide and the iodide are much less soluble in water and in ammonium hydroxide than the chloride.

The salts were prepared by adding equivalent amounts of sodium bromide or sodium iodide to a 0·003 % solution of silver nitrate. The pH was then regulated with weak ammonia and sections immersed in the solutions for 16 hr. at 58° C. In no case was any staining obtained. This may have been due either to the low solubilities in water and ammonium hydroxide or to some other property of these salts. The latter seems not unlikely, for the solubility of silver bromide at 100° C. (0·00037 g./100 c.c.) is considerably greater than the solubility of silver chloride at 10° C. (0·000089 g./100 c.c.) and both the bromide and iodide are slightly soluble in ammonia.

Many other silver salts were prepared by the addition of equivalent amounts of the appropriate sodium salt to 0·003 % silver nitrate. These can be classified into two main groups:

(1) Silver salts insoluble at this dilution. These included chromate, ferricyanide, and thiocyanate. In all cases no stain was obtained, which was probably accounted for by the stability of these salts.

(2) Silver salts soluble at this dilution. These included phosphate, carbonate, citrate, fluoride, oxylate and tungstate. They, as was expected, gave results which were, in the main, indistinguishable from those obtained with silver nitrate.

(*d*) *Variations in alkali*

Ammonia was used in all the previous experiments, principally because of its close association with silver techniques and because it was a solvent for silver chloride.

In a series of experiments it was replaced by other alkalis, namely, pyridine, ethylamine and sodium hydroxide.

With both pyridine and ethylamine results were obtained which were similar to those where ammonia had been used, except that the final result was redder and the nerve fibres did not contrast so well with the background. With sodium hydroxide, which does not dissolve silver chloride, some positive results were obtained. A few of these were almost as good as with ammonia, but it was obvious that it was difficult to control, small differences in pH producing a profound effect which was not obtained with ammonia. It was thought at first that the pH would remain more constant with sodium hydroxide but it was found to fall in 16 hr. at 58° C. from 7·6 to 6·5. Despite this, and contrary to the findings with ammonia, the results were not granular or greenish in colour.

Because of the difficulty in controlling the results with sodium hydroxide and the final colour of the sections with pyridine and ethylamine, these alkalis were discarded in favour of ammonium hydroxide.

(*e*) *Attempts at increasing the sensitivity of the stain*

(i) *Mechanism of silver chloride action.* Certain features of the silver chloride-ammonia stain which were discovered cast some light on the mechanism of its action.

(1) The sections are light brown in colour when removed from the impregnating solution. This suggests, as was found by Holmes (1943), that the silver was reduced by the tissue and was confirmed by omitting the developing bath, washing the sections and gold toning. Despite the absence of development the sections showed a considerable degree of staining which was less than that obtained when development was practised.

(2) *Double impregnation.* A further test was to impregnate sections in two or more baths of the standard stain each for 16 hr. at 58° C. without intermediate development. This process failed to produce any marked increase in the intensity of staining suggesting that the silver acceptors, presumably reducing agents, in the tissue had been saturated at the first impregnation. This was in marked contrast to Silver's (1942*b*) findings which led him to suggest that negatively charged silver micelles were deposited on positively charged proteins, and that the process was a continuous one and could be carried on till large quantities of silver had been deposited. Just how this was achieved is not clear, but the fact that the present findings agree with Holmes (1943) suggests that these methods work on a different principle. Tests carried out with physical developers of different types prior to gold toning showed that the initial impregnation could be intensified in this manner. The deposition of extra silver produced a granular effect on the surface of the fibres increasing their apparent thickness. This was in marked contrast to the microscopically agranular deposit which resulted from the standard method.

(3) In no case had it been possible, either by altering the fixative or the constituents of the stain, to obtain an impregnation of every nervous structure, especially in the cerebral cortex and in the case of olfactory and postganglionic autonomic nerve fibres.

(ii) *Reducing power of the tissues and sensitivity.* Because of this latter fact and the evidence pointing to the reducing power of the tissue as the principal agent, certain lines of investigation for increasing the sensitivity were attempted.

(1) To increase the sensitivity of the silver chloride by light and by the use of traces of mustard oil as in photographic emulsion sensitization.

Exposing the solution to light during impregnation destroyed the stain and the addition of mustard oil in minute traces produced no significant result.

(2) Three reducing substances were considered likely participants in the reaction: (*a*) sulphidryl groups; (*b*) ascorbic acid; (*c*) aldehydes.

Sections of tissues were treated with potassium cyanide solutions of varying strengths with a view to unmasking sulphidryl groups. At some concentrations of KCN, notably $<1\%$, there was some improvement in the stain, but treatment with alkaline solutions of the same pH produced similar results. It was therefore presumed that no unmasked sulphidryl groups were present in the sections or that sulphidryl groups were not taking part in the reaction.

Other sections were treated prior to impregnation with thioglycollic acid and with

a view to breaking S-S linkages and producing sulphidryl groups. This likewise produced no noticeable change.

It was therefore considered that SH groups were not responsible for the reaction.

In another series of tests sections were treated with ascorbic acid by immersion in solutions of various strengths for different times. It seemed that in some cases the stain was improved, but this was not particularly obvious and no conclusion could be drawn.

It was thought likely, in view of the above tests, that aldehydes were responsible in some measure at least. This view has recently been substantiated by Hsu-Mu Liang (1947), who has stained fresh and fixed nerve fibres with Schiff's reagent.

(3) Cajal, in some of his methods to demonstrate the finer structures of the nervous system (boutons), used chloral hydrate as a fixative. De Castro, in modifications of Cajal's methods, made use of both chloral hydrate and urethane in fixing fluids. The significance of these substances is not known, but empirical tests were made of the result of adding small quantities (*c.* 0·1 %) to the impregnating solution.

In the case of chloral hydrate the results were impaired and the silver chloride in suspension was partially reduced. With urethane no significant change was noticed, though Green (1948) has found that the addition of this substance to protargol improves the staining of fine fibres in the cerebral cortex.

(*f*) *Copper*

Bodian (1936) makes use of the addition of metallic copper to the protargol solutions. This is said to 'activate' the protargol. Holmes (1943) has demonstrated that the copper produces a steady fall in the pH of the protargol, and presumes that the changing pH allows the solution at some time to reach the optimum for staining different nervous tissues. This might account for the fact, mentioned above, that when metallic copper is added to the silver chloride-ammonia stain a higher initial pH is required to obtain adequate staining. This can be only a partial explanation with the present stain, because the addition of metallic copper to the solution, though requiring a higher initial pH for adequate staining, produces a stain whose characteristics are quite different from those of the simple stain.

This is noticeable at the end of impregnation when the sections are seen to be stained a reddish brown colour in contrast with the light brown colour normally seen. This reddish brown colour is principally concentrated in the nervous tissues, bone and muscle, and though other tissues may appear stained after gold toning, especially when the treatment with oxalic acid is prolonged beyond 3–5 min., the nerve fibres are everywhere more intensely stained. The colour range which is normally between red nuclei and muscle fibres and purple to black nerve fibres has, with copper, turned to one of light grey muscle fibres, grey nuclei with black nucleoli and black nerve fibres.

It would seem then that the addition of metallic copper to the stain, while removing much silver from the solution, increases the specificity of the reaction and alters the nature of the substance deposited in the tissue. This occurs even when the pH with copper does not fall below 7·3, at which point control sections in the standard solution without copper show the usual distribution of staining in the tissues with

the normal colour range. This seems to indicate that it is not simply the change in pH which is important, but suggests that the copper which passes into solution is in some way concerned with the impregnation.

To test this minute traces of various copper salts were added to the standard staining solutions. In all cases no specific staining could be obtained, and a similar result was found when weak solutions of copper hydroxide in ammonia were used in place of the silver solution.

The part played by the copper is not at all clear, but it is known that the addition of small quantities of the salts of heavy metals, including copper, to photographic emulsions increases their sensitivity to light by acting as centres of reduction or facilitating the formation of such centres.

(*g*) *Fixation, decalcification, etc.*

The part played by the fixative employed for treating the fresh tissue prior to the use of Bodian's method has been examined by many authors (Bodian, 1937; Davenport & Kline, 1938). With the present stain a wide range of fixatives has been employed, and good results have been obtained with formalin (10 %), Bouin, Carnoy (without mercuric chloride) and acetic (5 %)-formol (5 %)-alcohol (70 %). With Bouin-fixed material the picric acid is removed by means of several changes of 70 % alcohol containing 2 % 0·880 ammonium hydroxide. This step seems to improve the staining properties considerably, and has been used with advantage after acetic-formol-alcohol and Carnoy fixation.

For peripheral nerves the best results have been obtained with Carnoy and acetic-formol-alcohol (Pl. 1, figs. 1–3), while brain and spinal cord sections are well stained after formol fixation (Pl. 1, fig. 4) (especially with 10 % chloral hydrate (Pl. 1, fig. 5). In the spinal cord boutons have been regularly demonstrated after formol fixation, but a strange reciprocal staining has been noticed, some of the cells in a single section of the spinal cord demonstrating neurofibrils clearly but few boutons, while others show few cytoplasmic structures and a large number of boutons. No exclusive anatomical distribution of these two kinds of cell could be determined, and it seems likely therefore that there may be a variation in the distribution of the silver aeceptors in different functional states of the cell or in different types of cell. It is obvious that changes in pH of the stain cannot be responsible for the differences seen here, as Silver (1942*b*) has suggested.

With all the fixatives there is a tendency, in sections of the central nervous system, for the axons and dendrites of nerve cells to be stained but only the nuclei of the cell bodies to show up clearly. There are certain interesting exceptions where the cell bodies are stained with considerable regularity. These include all motor neurones, i.e. anterior horn cells and motor cranial nerve nuclei and the giant pyramidal cells of Betz. All root ganglion cells are stained as are the cells of the mesencephalic root of the trigeminal nerve. It would seem that all these cells have certain chemical properties in common, containing in moderate amount a silver acceptor which is a reducing agent, probably an aldehyde.

Decalcification has, where necessary, been carried out by means of Bensley's decalcifying fluid (50 % formic acid and 20 % sodium citrate in equal parts) which

was suggested by Green (1947*b*), who found that the use of this fluid improved the subsequent impregnation. This has been confirmed with the present stain, and pieces of tissue were routinely passed through this fluid before dehydration and embedding.

(*h*) *Development*

The developing solution used in all these tests was: hydroquinone, 1 g.; sodium sulphite cryst., 10 g.; water to 100 ml.

This solution has been found valuable in previous tests, and though it may be that the high concentration of sodium sulphite dissolves out some unreduced silver the end result is better with this concentration than with lower percentages of sodium sulphite. Whether this is a result of the pH or merely of the sulphite is not known, but Holmes (1943), in contrast to Silver (1942*b*), is of the opinion that the pH does not materially affect the stain. The temperature of development is of some significance for with temperatures below 15° C. hydroquinone acts slowly and incompletely.

Final method

(1) Pieces of tissue fixed in formalin, Bouin, Carnoy (without mercuric chloride) acetic-formol-alcohol, or acetic alcohol are treated for a few hours in 2% 0·880 ammonia in 70% alcohol, passed (if required) through Bensley's decalcifier for sufficient time to allow penetration, washed thoroughly in water, dehydrated and embedded in paraffin.

(2) Paraffin sections mounted on slides with albumen are placed in the following solution for 16 hr. at 58° C. in the dark: 0·1% silver nitrate, 2·9 or 3 ml.; distilled water, 100 ml.; mix thoroughly and add 0·1% sodium chloride (analytical), 1 ml.

Mix thoroughly and bring to pH 7·8 (Johnston Comparator paper 6883) by adding a few drops of very dilute ammonia. A very little of the silver chloride suspension is dissolved by this, but the appearance does not change materially. The solution should be made up just before use and not exposed to bright light for long.

(3) Remove the sections and place directly in the following solution for 5 min. at 18–20° C.: hydroquinone, 1 g.; sodium sulphite cryst. 10 g.; water to 100 ml.

(4) Wash well in tap water.

(5) Pass through distilled water into 0·5% gold chloride for 10 min.

(6) Wash sections in distilled water, two changes, for 1 min.

(7) Reduce in oxalic acid 2% for 3–5 min. Usually it is not advisable to exceed 3 min., for non-nervous structures tend to develop up and no marked improvement in the nervous tissue is gained by prolonging the oxalic acid treatment.

(8) Wash well in running tap water.

(9) 5% hypo 3–5 min.

(10) Wash well, dehydrate, clear and cover.

Results. Nerve fibres purple to black. Nuclei red. Neurofibrils purple, keratin yellow. Bone cells black.

If it is desired to obtain black nerve fibres throughout, raise the pH of the impregnating fluid to 8·3 and add 5 g. of metallic copper in the form of narrow gauge wire to the solution, otherwise treat as before.

CONCLUSIONS

The series of experiments described above has brought to light certain characters of a process whereby nerve fibres may be stained. The most important feature is the reduction of a silver salt by some substance or substances present in the nerve fibres, the nuclei of all cells, the cytoplasm of certain groups of nerve cells, myofibrils, bone cells and many other situations. The exact nature of the reducing substance is unknown and it is unlikely that any single substance is responsible for the reaction in all the tissues mentioned above. At least there is no evidence to suggest that the stain is chemically specific even though it demonstrates the same structures with complete regularity.

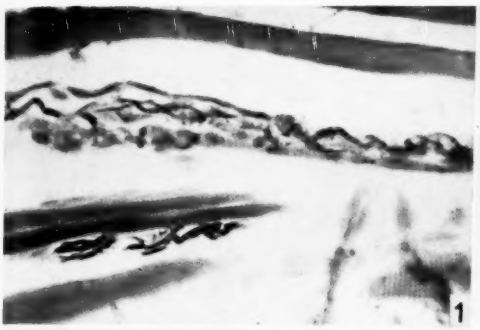
Many silver salts have been used in the tests, but only silver chloride has proved satisfactory, which is interesting in view of the presence of this substance in protargol. Why this salt should have produced results where silver iodide and silver bromide failed is not clear.

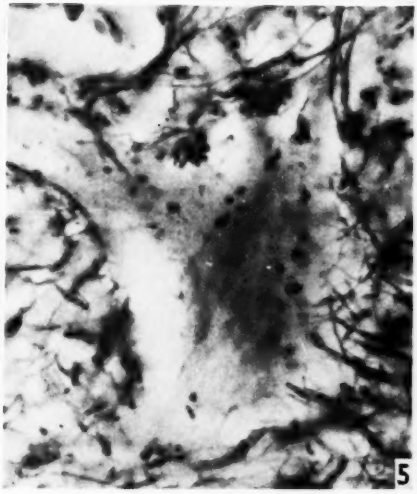
At the beginning certain criteria for a perfect silver method were set out. Of these the greater number have been fulfilled with one important exception, the failure to stain every nervous element. The outstanding failures were with the olfactory nerves, postganglionic autonomic nerve fibres and certain nerve cells. The two groups of fibres have one feature in common, the absence of a myelin sheath demonstrable by standard techniques. This would seem to suggest that the presence of such a sheath is associated with the ability to reduce silver chloride. The difference goes much deeper than this, for long before any myelin is present it is much more simple to stain the fibres which will develop a myelin sheath than those which will not. Also, no difficulty is encountered in staining the fine non-medullated parts of medullated nerve fibres (Pl. 1). It is undeniably true that in the earliest stages of development the staining of nerve fibres is a matter of some difficulty, but this is essentially the stage of rapid growth of the nerve-cell processes. Whatever the reason for this failure may be it indicates that these nerve fibres are in some chemical particular different from the others.

Despite these failures and the inability to intensify the reducing power of the nerve fibres prior to impregnation, the method described above has been proved to stain nerve fibres with great regularity while at the same time demonstrating many other elements but in such a way that they can be differentiated clearly from the nerves on account of differences in colour, a fact which cannot easily be demonstrated in monochrome reproductions.

The method has proved valuable with a large range of fixatives, is simple to prepare and the solutions are stable and can be made up in bulk ready for use. Only the weak ammonia solution may vary in concentration with the passage of time, but this is of no importance since the pH is tested on each occasion, and the addition of a greater or lesser quantity of water with the ammonia makes no difference within quite wide limits.

The silver chloride in the solution would seem to be the active principle in this method rather than that part of it which is dissolved by the ammonia, for when alkalis which do not dissolve silver chloride are used in place of ammonia, results of a similar character have been obtained. It would seem not unlikely in view of this





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and the fact that the stain is active only when the temperature is raised, that the small quantity of silver chloride in solution is gradually removed by the tissue and continually replaced by the fine suspension of silver chloride. This theory is borne out by the fact that the silver chloride gradually disappears from the solution when a large number of sections are impregnated in a relatively small volume of solution, but remains throughout, apparently unchanged, when only one or two sections are treated in the same volume.

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EXPLANATION OF PLATE

All the photomicrographs were taken on Ilford Microneg film without a filter. The sections which they portray were stained by the standard method without copper, but the contrast is less obvious in monochrome than in the originals.

Fig. 1. Part of a muscle spindle (mouse). Fixative, acetic-formol-alcohol. 10μ.

Fig. 2. Motor nerve endings (mouse) cut tangentially on the surface of two muscle fibres. Fixative, acetic-formol-alcohol. 10μ.

Fig. 3. Section through the organ of Corti (mouse) showing the nerve fibres crossing the canal. Fixative acetic-formol-alcohol. 15μ.

Fig. 4. Section of a giant pyramidal cell from the motor area of the cerebral cortex (dog.) Fixative, 4% formaldehyde. 10μ.

Fig. 5. A section tangential to a cell of the intermedio-medial group in the spinal cord showing boutons. Fixative, 10% chloral hydrate in 4% formaldehyde. 10μ.

THE RELATION OF THE DIRECTION OF THE MENTAL FORAMEN TO THE GROWTH OF THE HUMAN MANDIBLE

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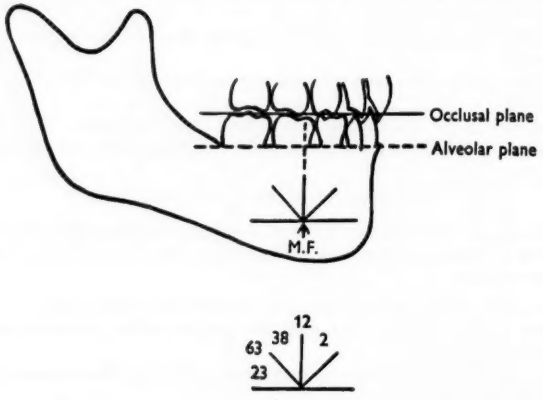
The peculiar direction of the mental foramen in the human mandible has long been noted by anatomists: John Hunter (1771), in his classic account of the growth of the jaws and palate, was clearly aware of it. The canal which connects the foramen with the inferior dental canal curves not only laterally (Pl. 1, fig. 1), but also markedly upwards and backwards (Pl. 1, fig. 2), so that the foramen points upwards and backwards, presenting a grooving or smoothing-out of its otherwise sharp margin in the postero-superior quadrant (Pl. 1, fig. 3). It has also been noted that at birth the grooving is inclined upwards and forwards (Pl. 1, fig. 4), the adult configuration being rapidly acquired in the first few years of life (Pl. 1, fig. 5). Brash (1924) likened the change in direction of the grooving to similar appearances in the growth of nutrient foramina, but, apart from his suggestion, little attention appears to have been paid to the problem. He also pointed out that the opening of the mental foramen moves backwards relative to the teeth during the years of growth.

Although it is not overtly stated, the direction of the foramen is usually described with the body of the mandible in the horizontal position. For the purpose of this study an imaginary line, parallel with the alveolar plane, has been employed to bisect the foramen, the angle of the grooving being referred to this base-line (Pl. 1, fig. 6). Upon this basis, it was found that the majority of foramina, after the age of 5 years, point at least upwards, and usually backwards as well (Text-fig. 1). In twenty mandibles from the newborn the direction was always upwards and forwards.



Text-fig. 1. Tracing of strict lateral view of an adult human mandible, showing alveolar plane and method of estimating direction of mental foramen. The figures record the number of foramina, right and left, directed approximately along the axes indicated in sixty-nine adult human skulls.

Dissections of the mental nerve, on both sides, in forty-five adult subjects have established the following facts:

(*a*) The largest element of the mental neurovascular bundle was the mental nerve, which is therefore presumably largely responsible for the grooving.

(*b*) The first few millimetres of the nerve always lay in the groove.

(*c*) The trunk of the nerve was usually somewhat tortuous.

(*d*) The classical description of the nerve, as given by Hovelacque (1927) and other authors, was confirmed: the branches fanned out towards the lower lip, the most posterior supplying the angle of the mouth, and the rest passing largely to the mucous membrane and glands, comparatively few reaching the skin of the chin (Pl. 1, fig. 7).

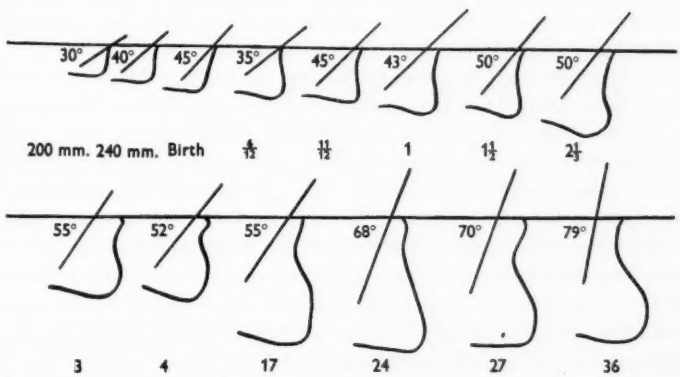
Dissections of the mental nerve in ten foetuses, from 140 mm. to full term (Pl. 2, fig. 8) revealed a similar distribution, but there were two important differences observed:

(*a*) The nerve was never tortuous.

(*b*) Its direction was distinctly forwards and somewhat upwards, corresponding with the foramen's direction in the neonatal mandible.

Material for dissection between the extremes of life is difficult to obtain, and to investigate the sequence of changes between infancy and old age recourse was had to a teleradiographic technique.

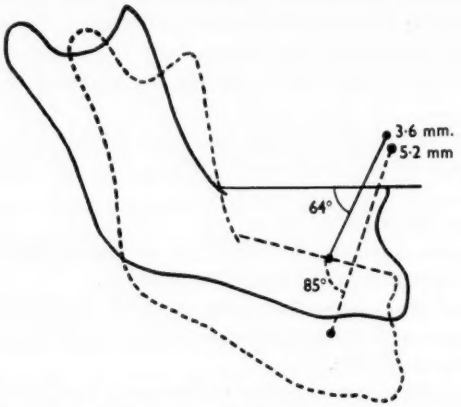
Upon the basis of the observed course of the nerve towards the skin around the angle of the mouth, this point was taken as a landmark in the living and indicated by attaching to it a lead shot. To minimize distortion, lateral exposures at 6 ft. were made. Tracings were made from the films, the centre of the foramen being joined to the centre of the lead shot, and the angle measured between this line and the alveolar plane (Pl. 2, fig. 9). A series of foetal and adult heads examined by the same method, as well as by dissection and photography, confirmed the accuracy of the method. A comparison of the results (Text-fig. 2) indicated that this angle becomes less acute in the first few years of life, and that the change is continued progressively but more slowly until adolescence.



Text-fig. 2. A comparative series showing the relation of the axis of the mental nerve to the alveolar plane and profile of the chin at various ages.

Nevertheless, these methods did not indicate a swing in the axis of the nerve into a backward direction in adults, and to account fully for the change in direction in the foramen from newborn to adult, some further factor was evidently involved.

Lateral teleradiographs of eight adults were obtained with the mouth closed as before and also widely opened. Comparison of the oral angle—mental foramen axis, in these two positions, added two further facts (Pl. 2, fig. 10 and Text-fig. 3).



Text-fig. 3. Tracings of lateral teleradiographs of the same subject with mouth open and closed. Note increase of the distance from mental foramen to oral angle in the open position.

(*a*) The axis becomes more vertical upon opening the mouth, but still did not incline backwards relative to the alveolar plane.

(*b*) The angle of the mouth moves considerably upwards and backwards relative to the foramen in the open position, an average of the eight cases showing a 40 % increase.

These findings suggest that in adults the mental nerve must be, in the closed or resting position of the mandible, considerably longer than a direct course from the foramen to the soft tissues supplied, and that it must therefore be tortuous, the condition regularly encountered in the dissecting room.

It appears therefore that two factors may be involved in the change of direction of the mental foramen: first, a functional variation in the distance from the foramen of exit to the soft parts supplied by the nerve, and, secondly, a relative difference between the growth of the mandibular and alveolar parts of the bone, and therefore between the foramen and the same soft tissues. The latter relationship is involved in the development of the chin, and it will be noted that the backward inclination of the foramen occurs approximately at the time of the development of the human chin.

Reference was therefore made to records of fossil man, and the modern condition of the foramen was found to exist in those forms which presented a prominent chin. In the hominids, which possessed little or no chin, the foramen was usually double or multiple, and so not directly comparable. In such cases, most of the foramina pointed forwards and upwards, but a posterior one was often directed upwards and backwards, perhaps serving to transmit the posterior branch of the nerve to the

angle of the mouth. In fossil jaws of children these multiple foramina appear to be all directed upwards and forwards, as in some fossil apes. However, precise information is difficult to obtain, and the nature of the fossil material often precludes dogmatic statement. It is hoped to report more fully upon this problem at a later date.

As regards the comparative anatomy of the foramen, inspection of nearly 400 vertebrate skulls, representative of all Orders, from teleosts to primates, has revealed great variety in its appearance. It is often multiple, and in this case one or two more posteriorly situated foramina may be grooved upwards and backwards. This is the condition in most primates except the anthropomorphs, which, like most fossil apes, present a single, forwardly directed foramen, sometimes duplicated. But the gorilla, alone of chinless forms, presents a single, usually backwardly directed foramen. Unfortunately, there has been no opportunity as yet to dissect the mental nerve in the gorilla.

To sum up these findings, it may be said that, when single, and so comparable with the recent human condition, the foramen is directed upwards and forwards, a fact which may be related to chinlessness or alveolar prognathism. The phylogenetic evidence is thus in agreement with the ontogeny of the human mental foramen; but the growth changes which produced a chin do not seem to provide a complete explanation of the change in direction of the foramen, and it is concluded that the functional factor of jaw movements is also involved.

These views are not in conflict with those expressed by Brash (1924). Condylar growth of the mandible would appear to alter the relation between the mental foramen and the area supplied by the mental nerve, thus altering the angle at which the nerve emerges at successive ages; but the observations reported here suggest that mandibular growth alone does not adequately explain these changes.

SUMMARY

In conclusion, it may be said that, as the surface of the human mandible grows by deposition around the issuing mental nerve, a bony canal is built up, and in its changing direction it conforms to the habitual position of the mental nerve at successive ages, thus recording the movement and growth of the nerve and its area of supply relative to the foramen.

The author wishes to thank Prof. G. A. G. Mitchell for his interest and encouragement, Mr H. Gooding and Mr P. Howarth for technical assistance, Mr F. L. Newell and Miss D. V. Hughes for radiographic help, and Prof. Wood Jones, Mr Noon, Mr R. U. Sayce and Dr P. M. Butler for the opportunity of inspecting osteological material.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Horizontal section of the left side of the body of the adult human mandible seen from above. The mandibular and mental canals are exposed to show the lateral swerving of the mental canal towards its foramen.

Fig. 2. Coronal section of the body of the left half of the adult human mandible seen from behind. The exposed mental canal is shown to ascend to its foramen.

Fig. 3. Lateral view of the right mental foramen. The alveolar plane is horizontal. Note the grooving of the otherwise sharp margin in the postero-superior quadrant.

Fig. 4. Right lateral view of a neonatal mandible, showing upward and forward direction of the mental foramen (indicated by black thread). Note poor development of chin.

Fig. 5. Right lateral view of 2½-year-old mandible, showing upward and backward direction of the mental foramen (indicated by black thread). Note completion of milk dentition and development of chin.

Fig. 6. Convention adopted for recording direction of the mental foramen in human mandibles. The horizontal line drawn through the centre of the foramen is parallel to the alveolar plane.

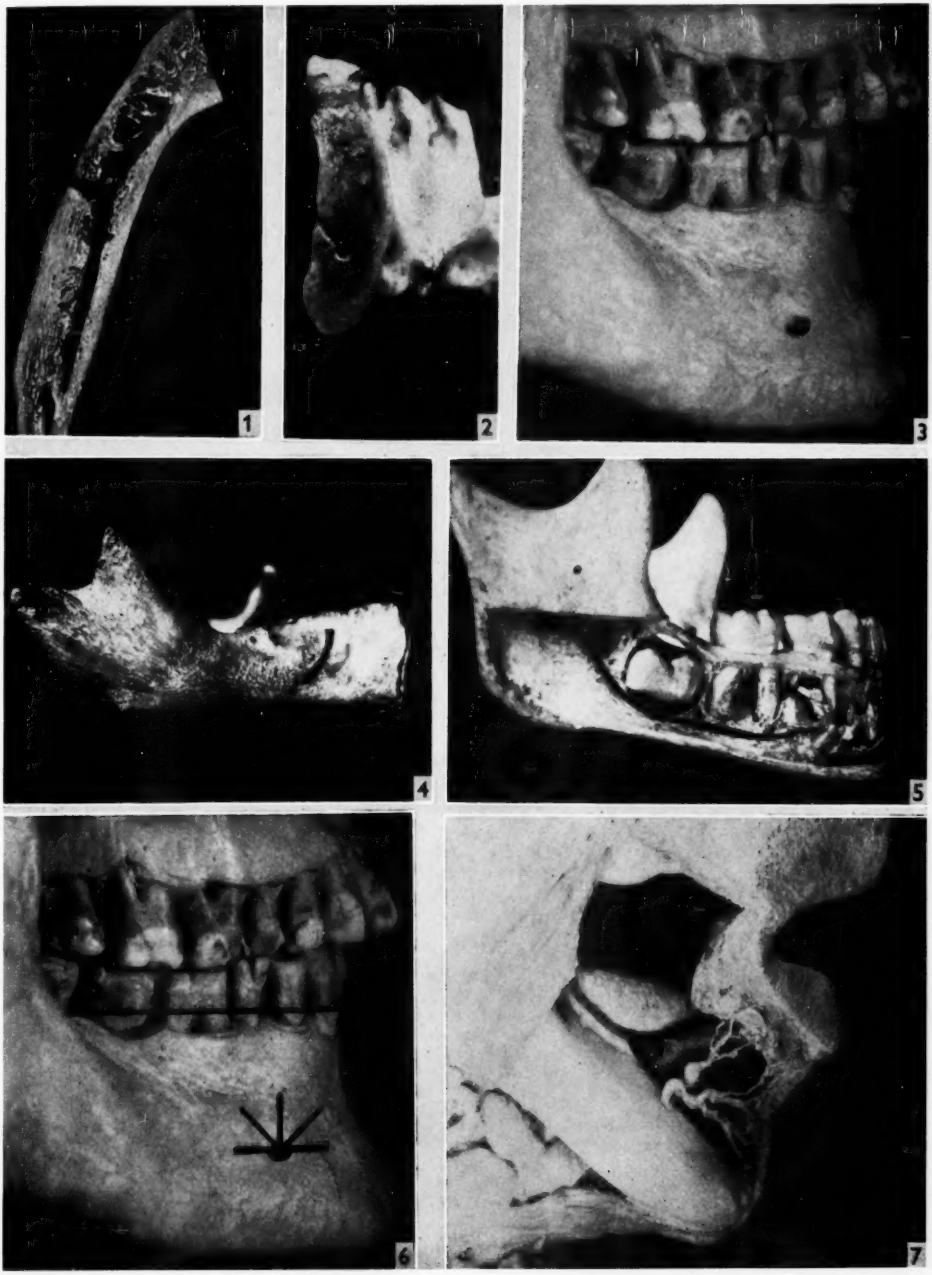
Fig. 7. Exposure of the left mental nerve in a dissecting room cadaver, showing tortuosity of main trunk, fanning out of the branches towards the lower lip, and position of the nerve at emergence.

Plate 2

Fig. 8. Exposure of mental nerve in 7-months foetus. Note lack of tortuosity and forward traverse of nerve towards the lower lip and angle of the mouth.

Fig. 9. Lateral teleradiograph of mental region of a 20-year-old male. The angle of the mouth is indicated by a lead shot, and the mental foramen, difficult to see in reproductions, lies in the small square. The horizontal line represents the alveolar plane.

Fig. 10. Lateral teleradiographs of a 24-year-old male with mouth open and closed. The oral angle, mental foramen, and alveolar plane are indicated as in Fig. 9.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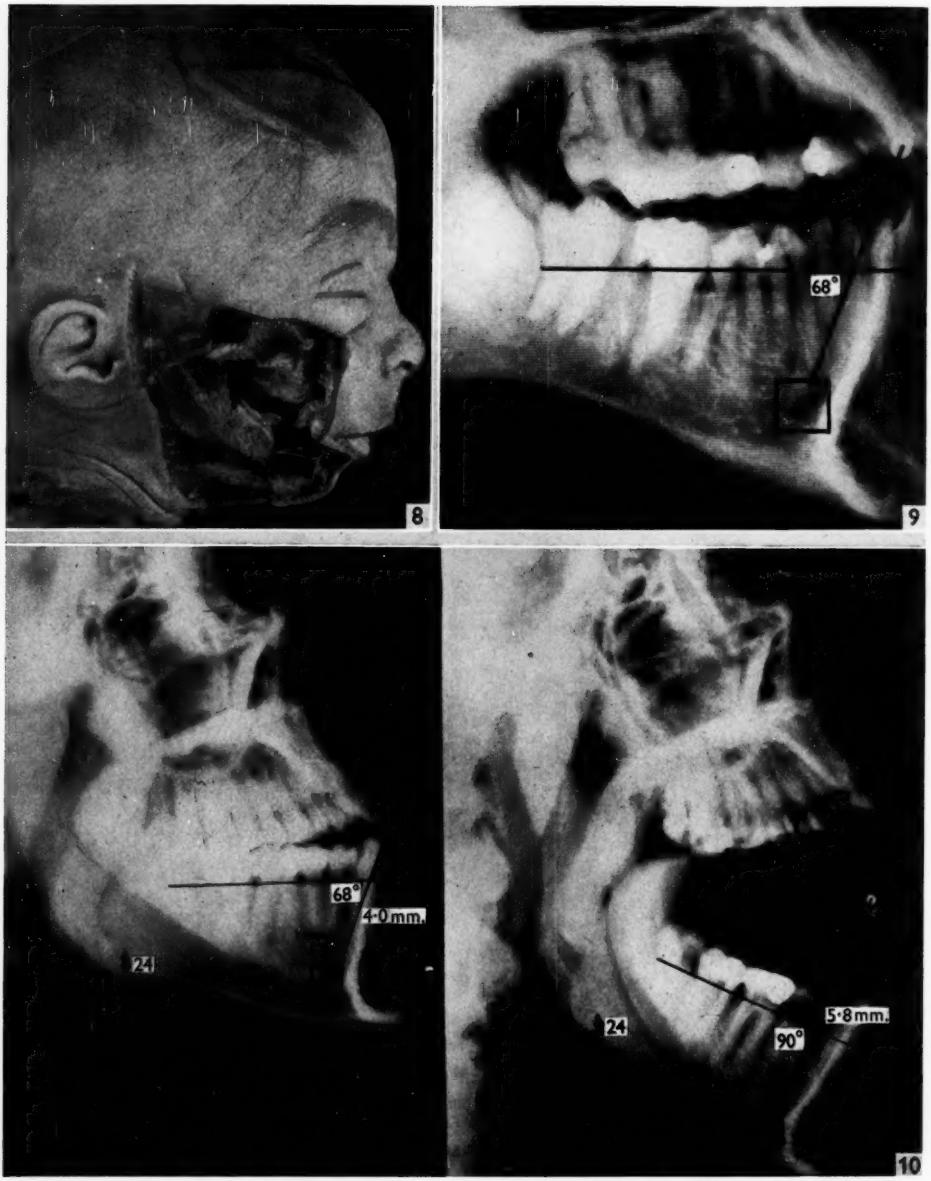

WARWICK—THE DIRECTION OF THE MENTAL FORAMEN

A DESCRIPTION OF THE CORONARY ARTERIES IN DIPNOAN FISHES AND SOME REMARKS ON THEIR IMPORTANCE FROM THE EVOLUTIONARY STANDPOINT

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INTRODUCTION

The similarity of the distribution of the coronary arteries in some fishes, on the one hand, and in the higher vertebrates on the other, has frequently been remarked upon. The view that the coronary system is homologous throughout the vertebrate series has been put forward by Grant & Regnier (1926) and by O'Donoghue & Abbott (1928). The latter authors have suggested that the alternative interpretation, which regards the coronary arteries of mammals as *vasa vasorum*, arises from the fact that too great emphasis has been placed upon the anomalous condition seen in certain anurous Amphibia in which these arteries are absent in the adult.

In a study of the frog's heart the present writer (Foxon, 1947) drew attention to the very reduced condition of the coronary arteries in frogs and toads, and pointed out that the evolutionary position of the heart of the modern Amphibia must be regarded as one of a high degree of specialization, thus reinforcing the view of O'Donoghue & Abbott (1928). Therefore, in any attempt to trace the homologies of the coronary vessels in fishes and in higher vertebrates, the modern Amphibia can, in general, be neglected.

When the coronary arteries of the fishes are considered it is seen that, in those so far described, the blood for this system is drawn from some part of the efferent branchial system, for its vessels are those which contain oxygenated blood. In general it may be said that this blood is derived through the hypobranchial arterial vessels and may be drawn either: (i) from the more anterior region of the ventral ends of the efferent loops, or (ii) from a more posterior origin in the region of the subclavian arteries. In some instances a combination of both origins for the vessels supplying the heart is found.

In the first category, where the coronary arteries are supplied from the more anterior region, they approach the heart along the ventral aorta and conus arteriosus. It is here that in certain fishes a distribution of the coronary vessels remarkably similar to that of the higher vertebrates has been found. In the second category the coronary arteries usually approach the heart from the posterior region of the pericardial cavity and are distributed over the heart from the caudal end.

However, even if the great similarity in distribution of coronary arteries over the heart in certain fishes and higher vertebrates has been recognized and its significance appreciated, what has not yet been convincingly demonstrated is how such a system, connected, as it must be, with the efferent system in fishes, could have its origin transferred to the vessels leaving the heart, that is, to the equivalent of the afferent branchial system. For the vessels leaving the heart, in the Tetrapoda, are to be

regarded, in their proximal portions at least, as homologous with the afferent arteries in fishes. O'Donoghue & Abbott (1928) have suggested that when the ventral aorta began to carry oxygenated blood, as a result of lung breathing perhaps, a new connexion was established between the cardiac end of the ventral aorta and the pre-existing coronary vessels, but this implies a possible functional discontinuity.

On looking at the matter more closely it became apparent that our knowledge of the coronary circulation of the existing Dipnoan fishes, which might reasonably be expected to suggest a solution to this problem, was incomplete and that further study might be well repaid. This has proved to be so and has provided the most satisfactory evidence yet obtained of the homology of the coronary arteries throughout the vertebrate series.

It may, perhaps, be pointed out that the heart and the branchial circulation in Dipnoi show a degree of structure and specialization unique in fishes; this may be summarized as follows:

The atrium, ventricle and conus arteriosus are partitioned, in various degrees of completeness in the three genera, into right and left parts, foreshadowing the division completed only in Crocodilia, birds and mammals. This division is least complete in *Ceratodus* and most complete in *Lepidosiren*, *Protopterus* occupying an intermediate position. Correlated with this division of the heart the blood from the lungs is returned, not with the general venous blood from the rest of the body, but separately into the left division of the atrium. The division of the ventricle and conus is such that in *Lepidosiren*, at least, the blood derived from the lungs probably passes, with only a small degree of mixture with venous blood, into the two most anterior afferent branchial arteries. It may also be noted that the afferent arteries take their origin almost directly from the anterior end of the conus, the ventral aorta being so shortened as to be unrecognizable.

In *Ceratodus* all the afferent vessels break down into gill capillaries which rejoin to form efferent vessels. In *Protopterus* the first and second branchial arches are devoid of gill lamellae and the vessels pass round the arches uninterruptedly, but the last two arches bear gills and the afferent vessels here break down to form capillaries in the usual way. In *Lepidosiren* all the arches bear small gill filaments but, nevertheless, the arterial arch can be traced round all the branchial arches completely and thus gill capillaries are not interposed between afferent and efferent arteries in the usual way; each arch is single and complete, its ventral portion may be called afferent and its dorsal portion efferent. The gill filaments themselves are feebly supplied with blood, and Fullarton (1931) has brought forward evidence to support the contention that they play little part in respiratory exchange which, it is concluded, is performed almost entirely by the lungs. The lungs are supplied with blood from the two posterior efferent vessels.

From this brief summary it will be seen that there is probably considerable difference in the methods of oxygenation of the blood in the various genera of Dipnoi, and it is to be expected that, as the muscles of the heart will need highly oxygenated blood, the source of this blood will also vary in a manner which may be correlated with the difference in the mode of oxygenation.

CORONARY ARTERIES IN DIPNOI

The distribution of the coronary arteries over the heart has been described in *Ceratodus* by Spencer (1892), and in *Lepidosiren* by Robertson (1913). So far as I have been able to ascertain, these arteries have not been described in *Protopterus*; they are certainly not mentioned in the classical description of the blood system of this fish by Parker (1892).

The origin of the coronary arteries from the hypobranchial arterial system and the relations of the hypobranchial system to efferent and afferent systems has only been described for *Ceratodus* by Spencer (1892). Here a typical fish-like condition is seen, with a supply from the anterior hypobranchial system.

I have been able to trace the origin of the coronary arteries with certainty in *Lepidosiren*. In *Protopterus* coronary arteries undoubtedly occur, but it is not yet possible to describe the hypobranchial system. It is convenient to deal with these two fishes separately.

(*a*) *Coronary arteries in* Lepidosiren

Material. I have to thank Prof. C. M. Yonge and Mr C. W. Parsons for allowing me to use specimens of *Lepidosiren* from the collections in their care at the Zoology Department, Glasgow University.

The anterior parts of two large and one small fish have been available; these had all been preserved for several years. Mr Parsons kindly sent me, fresh, the body of a small specimen which had died in captivity. Two very small adults which were two of those hatched in the Zoology Department at Glasgow in 1935 (see Parsons, 1935) were available for sectioning. The main method of investigation, has, however, been dissection. It was found impossible to make the vessels of the preserved specimens more conspicuous by injection. At a late stage in the course of this investigation, Mr Parsons kindly sent me a whole specimen which died in captivity at Glasgow. This specimen which measured 40 cm. in length, a rather small adult, was injected by Mr Parsons and Dr Steedman with latex before being sent to me, and has proved very useful for the clarification of important points.

Description. In describing the heart of *Lepidosiren*, Robertson (1913) says of the coronary arteries: 'Two little arteries are present one on each side of the bulbus cordis. The right vessel is the larger, it passes along the right wall of the distal part of the bulbus, dorsal to the transverse part, and then along the left wall of the proximal part, to the ventricle where it is distributed. The small left artery supplies the left dorsal wall of the distal part of the bulbus. The course of these little vessels anterior to their appearance on the side of the bulbus has not been traced, owing to the difficulty of dissecting the fibrous tissue in the region of the ventral aorta.'

I experienced the same difficulty with this tough fibrous tissue at the anterior end of the conus. (The term 'conus arteriosus' is used where Robertson wrote 'bulbus cordis' as being more in keeping with general usage, see Parsons, 1929.)

It soon became apparent that this tissue is too thick to allow satisfactory dissection of a vessel the course of which is unknown. However, since in all fishes which have coronary arteries coming from the anterior part of the pericardium these vessels must necessarily approach from the direction of the gills, it was decided to explore

thoroughly the region of the afferent branchial vessels in the hope that it would be possible to locate the coronary arteries anterior to the point at which they entered the pericardium. This method proved to be satisfactory, and although it has been impossible to prepare a dissection of 'Museum' quality it has been found possible, by piecing together evidence derived from the several specimens, to arrive at conclusions concerning the origin and course of the coronary arteries which are believed to be substantially correct.

The arrangement is shown in Fig. 1.

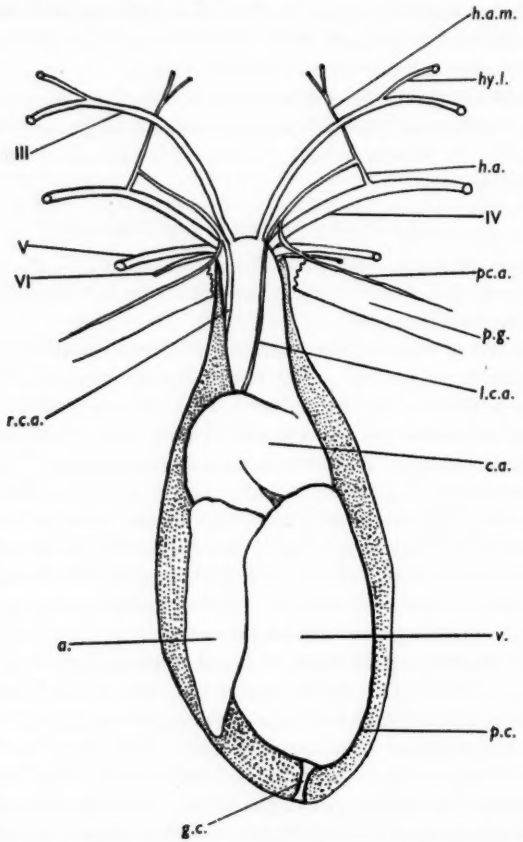

Fig. 1. Diagram of the heart of *Lepidosiren* and its coronary supply in ventral view to show the origin of the coronary arteries from the hypobranchial blood system.

a.	portion of atrium	*l.c.a.*	left coronary artery
c.a.	conus arteriosus	*p.c.*	pericardial cavity
g.c.	gubernaculum cordis (cardiac ligament)	*p.g.*	pectoral girdle
h.a.	hypobranchial artery	*pc.a.*	precoracoid division of hypobranchial artery
h.a.m.	branch of hypobranchial artery to muscles below buccal cavity	*r.c.a.*	right coronary artery
		v.	ventricle
hy.l.	branch of first (morphological third) arch to the hyoidean hemibranch	III, IV, V, VI.	arterial arches denoted by their morphological numbers

From the second afferent vessel (to the morphological fourth arch) on both sides at a distance approximately equal to the length of the distal part of the conus, a small vessel arises which runs in an anterior direction slightly converging to the mid-line. This vessel, which I call the hypobranchial artery, is about $\frac{1}{3}$, or slightly less, of the diameter of the afferent artery itself. The hypobranchial artery soon branches into two, a smaller branch running forwards into the muscles of the region of the floor of the mouth, a larger branch turning sharply towards the mid-line and running back to the region of the pericardium parallel to, and slightly ventral to, the second (morphological fourth) afferent vessel. Shortly before entering the fibrous tissue of the pericardium this vessel divides into two nearly equal branches. The slightly larger one turns outwards and runs along the anterior border of the coracoid bone. The smaller branch pierces the pericardial wall and continues on the side of the conus as the coronary artery.

The course of the coronary arteries within the pericardium has been described by Robertson, as previously stated. The specimens at my disposal show certain minor variations from Robertson's description. In particular, the left coronary artery, which she described as supplying the 'left dorsal wall of the distal part of the bulbus', in two of my specimens, including the freshly dead specimen, clearly ran on to the ventral surface of the distal part of the conus arteriosus and, looping forward again, ran anteriorly on the transverse part to disappear on the dorsal surface of the proximal part. In these cases it had a greater distribution than Robertson suggests. The right coronary artery I have found to run on the right dorsal portion of the distal part of the conus rather than directly down the right side. I cannot add anything further to Robertson's description.

An interesting variation was met with in the small adult specimen which was injected before being sent to me. The origin of the hypobranchial system was found to be as described above on the right side, but on the left side the origin was from the first (morphological third) arterial arch, i.e. the one that becomes the carotid in Tetrapoda. This is of some interest as it is the vessel from which the coronary vessel normally arises in the frog (*Rana temporaria*).

The precoracoid artery rapidly decreases in diameter, and though I have been unable to arrive at a definite conclusion concerning a possible anastomosis with the subclavian vessels, I am doubtful whether such a connexion exists. As will be made clear in the discussion, this is a point of considerable theoretical interest, and that the matter must remain in doubt is disappointing. Spencer (1892), in *Ceratodus*, found no trace, apparently, of a coracoid artery and, therefore, no evidence of any anastomosis between hypobranchial and subclavian systems.

In the fresh specimen sent to me by Mr Parsons shortly after it had died, it was clearly seen that there was a considerable colour difference between the blood remaining in the first two afferent vessels and the last two afferent vessels. The anterior vessels contained blood that was bright red and, therefore, presumably highly oxygenated, whereas, that in the posterior afferent vessels was of a much darker colour, although not nearly so dark as that in the veins, for example, of a freshly killed mammal. The colours were easily observed as the vessels are of a pale translucent appearance and the colour of the blood was not obscured in any way, for example, by pigment cells in the walls of the vessels. This observation is

placed on record as indicating that the separation of the blood in the heart of *Lepidosiren* into arterial and venous streams is accomplished in a manner which, though obviously incomplete, is probably of physiological significance.

(*b*) *Coronary arteries and cardiac ligaments in* Protopterus

Material. The material available to me has been, first, the head of a large specimen which had been preserved in the collections of Glasgow University for many years; for this I have to thank Prof. C. M. Yonge. Secondly, two young fresh specimens which died during early summer, 1948, in the aquarium of the Zoological Society of London, and for which I have to thank the Scientific Director, Dr Edward Hindle. Thirdly, through the kindness of Dr E. Trewavas, I have been able to examine the heart, *in situ*, of a large specimen of *Protopterus aethiopicus* (6 q 430) in the British Museum (Natural History).

Description. The two young *Protopterus*, lengths 15·5 and 18 cm. (approximately), received from the Regent's Park aquarium, had hearts measuring 7 and 10 mm. in length respectively. Both had met their death by fighting with their fellows and therefore had lost a certain amount of blood. Nevertheless, certain observations were possible in the fresh specimens. In neither instance was there any hint of difference in colour between the anterior and posterior afferent branchial vessels; and this would suggest that, at this stage at least, there can be little or no difference in the oxygenation of the blood in the various afferent vessels or in the two sides of the heart.

In the smaller fish no coronary artery could be seen on the conus. The heart was sectioned and no trace of any coronary vessel could be found in the sections.

In the larger fish it was at first thought that no coronary vessel was present, but some lymphoid material was seen to be present on the conus, much as is found in *Lepidosiren*, and close inspection of this revealed the presence of a very small vessel on the ventral side of the conus. However, nothing further of the distribution or relations of this vessel could be made out.

The specimen at the British Museum also has apparently a trace of a coronary vessel on the ventral surface of the conus beginning at the left side at the distal end of the conus and twisting ventrally over the straight distal portion of the conus.

The Glasgow *Protopterus*, however, gives a much clearer picture of this vessel. The heart of this animal is 3 cm. in length. There is a very clearly defined coronary artery running down at the right side of the distal part of the conus, giving small branches to its ventral wall; there is also a smaller vessel running on the left side of the conus. The small left vessel branches over the left distal part of the conus; the area served by the right vessel is much greater and, in fact, the vessel is still large where it meets the transverse portion of the conus. Here its course was difficult to follow in dissection, but appeared to be dorsal across the transverse portion, where it was obscured by the atrium, and then it reappeared on the ventral surface of the proximal portion running in the groove at the anterior end of the ventricle. Here it was smaller in diameter than before, branches presumably having been given off along its course. The general appearance is shown in Fig. 2.

A point of considerable interest revealed by this specimen is that the coronary

arteries do not follow the twist of the conus in the way they appear to do in *Lepidosiren*. In particular, no vessel could be seen following the large flexure of the transverse portion of the conus. This was so striking that it was decided to make serial sections of the 3 cm. heart just described. Sections cut transversely show clearly the course of the large right coronary artery which is shown in Fig. 2: that is, it runs posteriorly along the right side of the conus in its distal portion. It is not exactly lateral but very slightly dorsal on the conus wall. Where the conus makes its large transverse loop the coronary artery continues directly posteriorly, crossing

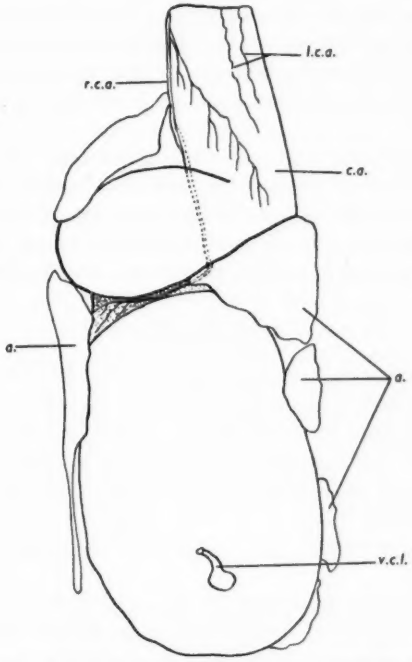

Fig. 2. Diagram of the heart of *Protopterus* from the ventral side, showing the distribution of the coronary arteries. The dotted lines indicate the course of the right coronary artery on the dorsal surface of the heart. *v.c.l.*, ventral cardiac ligament. Other abbreviations as in Fig. 1.

the transverse portion and becoming deeply embedded in the connective tissue which binds the conus, atrium and ventricles together in this region. Finally, it can be traced into the groove between the atrium and ventricles where it gives off many small branches. Few branches were seen to leave it in the more anterior part of its course.

Nothing definite can be stated about the origin of the coronary vessels in *Protopterus*, except that the supply is drawn from a hypobranchial system. In spite of the experience gained with the *Lepidosiren* material, I have been unable to convince myself by the dissections I have made, that I have found all the details of the hypobranchial system.

The ligaments attaching the wall of the ventricle to the pericardium, call for some comment.

In the specimen at the British Museum and in the specimen from Glasgow the ventral wall of the ventricle was found to be firmly attached to the pericardial wall by the ligament known as the gubernaculum cordis. This ligament was not apical, as is shown in the figure of a *Protopterus* heart given by Goodrich (1930, p. 552), but in both instances it was attached to the ventral surface of the ventricle, as shown in Fig. 2. In *Protopterus*, unlike *Lepidosiren*, a dorsal ligament, which is much finer than the ventral one, joins the ventricle with the dorsal wall of the pericardium. The ligament was present in the Glasgow specimen, and also I believe, in the British Museum specimen, but verification of this by dissection was not possible.

In the young specimens received from the Zoological Society's aquarium the ligaments were not so well developed, and their absence in the smaller specimen deserves mention.

In the larger of the two specimens only the dorsal ligament was present and this so fine as to have been overlooked at the first examination. The observations are interesting in suggesting that the cardiac ligaments may develop very late in ontogeny, a point of importance in view of some suggestions concerning ligaments advanced by Grant & Regnier (1926) and discussed later in this paper.

DISCUSSION

It is now possible to state that coronary arteries exist in all three Dipnoan genera. In *Lepidosiren* as described by Robertson (1913), in *Ceratodus* as described by Spencer (1892) and in *Protopterus* as described above. It will be noted that in all three the coronary supply is from the direction of the anterior end of the conus, and although the hearts of *Protopterus* and *Lepidosiren* are joined to the wall of the pericardium by various ligaments, these ligaments are not recorded as being vascularized and no trace of blood vessels approaching the heart along these routes has been noted in the work described above.

In all three genera the right coronary supply seems to be more important than the left. That this is so is recorded for *Lepidosiren* by Robertson, and for *Protopterus* above. For *Ceratodus* Spencer states that the coronary vessel arises from the right side only.

In *Ceratodus* a typical piscine circulation in the gill region is present, all the afferent vessels breaking down into gill capillaries from which the blood is returned through efferent vessels. It is from the ventral end of the more anterior of the two efferent vessels, between the second and third gill clefts that the hypobranchial system draws its blood supply. Thus oxygenated blood is supplied to the hypobranchial region including the muscular wall of the heart.

If we compare this condition with that in Elasmobranchs we find that, in general, in the latter group, where the efferent vessels form closed loops at their ventral ends, the hypobranchial arteries are derived from these ventral loops, but as a rule several loops have branches connecting them with the hypobranchial vessels. In addition, the hypobranchial system is sometimes connected posteriorly with the subclavian artery, but in Elasmobranchs this connexion is variable (see O'Donoghue & Abbott,

1928), and it would seem that the anterior connexion of the hypobranchial system is both more important and possibly more primitive. Passing to other groups of fishes, in the 'Shovel-nosed sturgeon', *Scaphirhynchus*, Danforth (1916) has described how the coronary system is connected to the subclavian artery. In the 'Spoonbill sturgeon', *Polyodon*, the coronary artery is derived from the most posterior of the efferent branchial vessels (Danforth, 1916). In the Teleostei several types of coronary supply have been recorded, including both anterior and posterior supplies. Grant & Regnier (1926) have put forward the hypothesis that the heart of such a Teleost as the Eel (*Anguilla*) shows a primitive condition. Here there is an anterior supply arising indirectly from the fourth efferent branchial vessel and a posterior supply which arises from parietal branches of the dorsal aorta supplying the pericardium. These authors bring into their arguments the cardiac ligaments, which in certain fishes join parts of the heart to the pericardial wall, and suggest that the vascular supply will approach the heart along all convenient routes. They go on to suggest that the course of evolution in the fishes has been towards simplification, both in the number of coronary arteries which are present, and in their origin.

I do not agree with this view. This matter cannot be debated at length here, but it would seem that the series of simplifications which Grant & Regnier (1926) suggest can be reversed and that it might be more easy, in the light of modern views on the evolution of fishes, to put forward the view that the anterior origin of the coronary arteries is the more primitive and that other supplies are specialized. It would seem clear from its ontogenetic development that the heart is at first a simple tube fixed in the pericardium by its two ends and that the flexures which arise later are due to the increase in length of the heart tube without corresponding increase in the length of the pericardium. If this is indeed so, then in so far as flexure of a tube bound to the pericardial wall by ligaments would be impossible, such ligaments should be regarded as later developments and in all probability indicative of a specialized condition.

Several points of interest in the coronary supply of *Lepidosiren* must now be commented upon.

First, the hypobranchial system is in all essentials, except one, similar to the hypobranchial systems of other fishes having an anterior origin for the coronary arteries. For example, these arteries have the same general pattern of distribution, supplying oxygenated blood to all parts of the sub-branchial region of the fish and to the heart muscles and pericardial area. The important exception that has to be noted is that this system, instead of being derived from efferent branchial vessels as in all other fishes, is connected with the second afferent vessel (morphological fourth) on both sides. But as has already been made clear, there is very good reason, both from study of structure and from observation, to suppose that this so-called afferent vessel is in reality carrying oxygenated blood.

Secondly, the hypobranchial system does not receive any additional contribution from the more posterior arches as it does in some other fishes. Thus it cannot convey deoxygenated blood which is on its way to the lungs through these two posterior arches.

Thirdly, it is possible to see how the transference of the hypobranchial connexions from efferent to afferent systems has been achieved. It is brought about simply by

the failure during development of the embryonic arterial arches to break down into capillaries in the gill septum. Thus the main hypobranchial arteries bear the same relationship to the gills in *Lepidosiren* as they do, for example, in *Ceratodus*, the only difference being that in the former, as the arterial arch is continuous through the gill, the hypobranchial artery connects with afferent portions of the arch which, however, happen, as we have seen, to carry oxygenated blood.

Two further points of interest emerge. First, the homology of the coronary arteries of fishes with those of higher vertebrates, already suggested by other authors, is clearly demonstrated, and there is no need to suggest, as did O'Donoghue & Abbott (1928), new connexions between pre-existing arteries and the ventral aorta. Secondly, the importance of the connexion of the hypobranchial arteries with the fourth morphological afferent and efferent vessels must be emphasized. This connexion is, in general, found in those fishes which have an anterior origin for the hypobranchial system; but the blood supply is frequently reinforced by extra connexions from other efferent vessels. For the efficient working of the coronary system it is essential that oxygenated blood only should be conveyed by the coronary arteries and it is a most extraordinary coincidence, if nothing else, that in *Lepidosiren* in which the more posterior vessels contain deoxygenated blood the only connexion between the coronary arteries and the blood supply to the gill is through the second afferent vessel which contains oxygenated blood. Moreover, this is the vessel which becomes the systemic arch in the tetrapods and from which the coronary artery or arteries are derived in Reptilia, although usually only from the right side (see Spalteholz, 1908). The Frog, as an example of the Amphibia, is somewhat different, since here the coronary vessel (which, however, as is generally agreed, has no function so far as blood supply to the myocardium of the ventricle is concerned) arises from the left carotid vessel (right vessel according to Grant & Regnier, 1926).

The position of *Ceratodus* is of interest, since here, where all the afferent vessels break down into capillaries in the gills and where presumably the whole circulation is more typically piscine, the connexion of the hypobranchial system is with the second gill cleft only.

To summarize, it may be said that the study of the Dipnoi and of *Lepidosiren*, in particular, shows that the coronary arteries are homologous throughout the vertebrate series and that the transference of the origin of these vessels from efferent to afferent branchial systems has come about by the failure during development of the arterial arches to break down into gill capillaries. In addition this development has kept in step with the partition of the heart into arterial and venous sides in such a way that, while connexion of the hypobranchial arteries (from which the coronary arteries take their origin) with the more posterior arterial arches is lost, the connexion with the second arch, which carries oxygenated blood, is retained. Finally, in the higher vertebrates various stages in the gradual movement of this connexion along this arch (which is now the systemic) can be traced (Spalteholz, 1908) until in the mammals the coronary arteries arise from the very base of the aorta.

SUMMARY

1. In *Lepidosiren* the following have been described: (*a*) the origin of the coronary arteries from the hypobranchial arterial system, (*b*) the connexion between the hypobranchial arterial system and the second (morphological fourth) arterial arch.

2. In *Protopterus* the occurrence of coronary arteries is described, but it has not been possible to trace their origin.

3. It is pointed out that the conditions seen in the Dipnoan fishes indicate how, in evolution, the origin of the hypobranchial arterial system may have been transferred from the efferent branchial system to the afferent branchial system.

4. Several authors have previously taken the view that the coronary arteries are homologous throughout the vertebrate series. The argument put forward here strongly supports this contention.

5. The occurrence and significance of the cardiac ligaments is commented on.

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ELECTROMYOGRAPHIC STUDY OF PATTERNS OF ACTIVITY OF THE ANTERIOR ABDOMINAL WALL MUSCLES IN MAN

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INTRODUCTION

Electrical potentials can be recorded from normal contracting muscle (the electromyogram or e.m.g.) by means of electrodes either inserted into the muscle, as with the concentric needle electrode (Adrian & Bronk, 1929), or connected to the surface of the muscle, e.g. the belly-tendon lead. An electrical action potential is produced whenever a normal muscle contracts. Conversely, when no action potentials can be detected in muscle, the muscle is at rest, i.e. completely relaxed (Adrian & Bronk, 1929; Lindsley, 1935; Weddell, Feinstein & Pattle, 1944). Complete relaxation of most muscles can usually be achieved voluntarily.

The motor unit, consisting of a single motor nerve fibre of the motor nerve, together with the bundle of muscle fibres which it innervates, is the physiological unit of muscle action (Sherrington, 1929). All the muscle fibres of the motor unit contract approximately simultaneously, and give rise to the motor unit action potential of normal muscle. When a muscle contracts more forcibly, more motor units are involved and their rate of discharge is higher. This means that more unitary action potentials can be detected in the electrical record and higher rates of discharge observed for the different units.

Surface electrodes placed on the skin over a muscle, such as the rectus abdominis, pick up the algebraic sum of the action potentials of all active motor units in the muscle, but the parts of the muscle more remote from the electrodes have a smaller effect than the adjacent parts. Thus, the electrical record is composite and represents the summated electrical activity of all the active motor units, with due regard to (i) their spatial distribution, relative to the recording electrodes, and (ii) the temporal dispersion of firing. Surface electrodes can usually be placed so that the action potentials are picked up from one muscle only, but for each particular muscle so studied it is essential to ensure that extraneous action potentials from adjacent muscles do not interfere with the record. With electrodes fixed over a given muscle, the electromyograms for different movements can be arranged in order of amount of activity, even though the force of contraction cannot be assessed quantitatively from the record. Increased force of contraction is reflected in the surface electromyogram by increased frequency and amplitude of the potentials. When the same pattern and amount of electrical activity is found in a muscle for two different movements, we shall say that the muscle is involved to the same extent in the two movements.

Superimposed on most records in this study is seen the electrocardiogram (e.c.g.),

the amplitude depending on the amplification chosen for each particular recording. The e.m.g. and e.c.g. records are, however, easily distinguishable; the presence of the e.c.g. does not confuse the interpretation of the e.m.g. record.

The transversus abdominis cannot be studied with surface electrodes, but the actions of this muscle should not be forgotten in discussing certain movements, especially those involving compression of the abdomen. We decided against studying this muscle with needle electrodes, the only possible technique, owing to the proximity of the peritoneum.

METHOD

The twenty-one subjects used in this investigation were all young adults, fifteen males and six females, ranging in age from 18 to 25 years. All subjects had flat or nearly flat abdomens. Obese or elderly subjects were not included in this series.

Six electromyograms were recorded simultaneously from different muscles, and in some experiments from different parts of the same muscles. An Ediswan standard 6-channel electroencephalograph was used, and all the records illustrated in the present paper were obtained with the ink-writer oscillographs incorporated in the machine. It is not necessary to use cathode-ray oscillographic recording for this type of study, since details of waveform are of no significance. The main features are amplitude and frequency, which are adequately recorded with the ink-writers.

Surface electrodes were used in all experiments, and were placed on the skin near the muscles of the anterior abdominal wall, as described below, and on some other muscles, viz. the sternomastoid and erector spinae, in certain experiments. The electrodes were silver, silver chloride, in a sodium chloride solution of the pattern used for electroencephalographic (e.e.g.) recording in our laboratory.

Electrode lay-out. Pairs of electrodes were placed symmetrically on the abdomen on each side of the mid-line in the following positions (Pl. 1):

(1) *On the rectus abdominis.* One electrode was placed over the upper part of the rectus abdominis between the xiphisternum and the umbilicus. The other was placed 10 cm. below the umbilicus at an equal distance from the mid-line. Care was taken to avoid the tendinous intersections in each case, though, with the type of e.e.g. electrode used by us (about 1 cm. in diameter), very little difference in the record was seen when an electrode was placed over an intersection.

In certain experiments different parts of the same rectus muscle were compared, four electrodes being placed over each rectus, in corresponding positions on each side of the mid-line, 6 cm. apart along the long axis of the muscle. Similar activity was found in the different segments for each movement studied.

The 'rectus electrodes' pick up only from the rectus muscle. They do not pick up from the external or internal oblique, nor from the transversus muscles. No muscle, apart from the rectus, underlies the electrodes. Furthermore, as shown later, the typical pattern of activity for straining involves the external and internal oblique and transversus muscles, but not the recti. This result could not have been obtained if there had been a large extraneous pick-up, at the rectus electrodes, from these other muscles.

(2) *On the external oblique.* A pair of electrodes was placed 3·5 cm. apart on each side in the flank, above the anterior half of the iliac crest, in an oblique line parallel

with the underlying external oblique muscle fibres. The electrodes do not record from the internal oblique on account of distance from that muscle and the low amplification used.

(3) *On the internal oblique.* A pair of electrodes was placed on each side over the triangle bounded by the lateral edge of the rectus sheath, the inguinal ligament and the line joining the anterior superior spine to the umbilicus. In this area the fibres of the internal oblique are separated from the skin only by the external oblique aponeurosis, and hence are accessible for study with surface electrodes. The electrodes were placed in a line parallel with the inguinal ligament, i.e. over those fibres which arch over the inguinal canal and are inserted into the conjoint tendon. References in this paper to the internal oblique muscle apply only to those fibres of it in the retro-aponeurotic triangle, and the behaviour of these fibres should not be regarded as an indication of the behaviour of the rest of the muscle.

It is possible that the internal oblique electrodes may pick up from the lowest fibres of the transversus abdominis. These fibres also arch over the inguinal canal and are conjoined with the internal oblique fibres. The similarity of origin, course, and insertion between these two sets of transversus and internal oblique fibres makes it likely that their function is also similar. For this reason no attempt is made to differentiate between them. It was thought possible that the cremaster and psoas muscles might contribute to the activity recorded from the internal oblique electrodes, but their contribution was found to be insignificant.

The amplitude of the recording from these electrodes is slightly diminished by the proximity of the electrodes to each other, 2·5 cm. compared with 3·5–10 cm. elsewhere. It is necessary to keep both electrodes as far away as possible from the rectus and external oblique fibres. For this reason the electrodes were placed 2·5 cm. apart. As the same amplification has been used in all six channels at any one time, the relative amount of activity in the internal oblique fibres is slightly greater than appears from the record.

When the activity from the corresponding muscles of both sides is compared, a small difference is commonly seen. This difference may be due to functional asymmetry: for example, the left rectus may predominate in one movement, and the right rectus in another. The electrodes are sited in corresponding areas of skin, measurements being made from the umbilicus, the mid-line and the anterior superior spine. Often the abdomen is not symmetrical, for example, the 'mid-line' and umbilicus may be 2–3 cm. to one side. Thus the electrodes may not be correspondingly placed over functionally or anatomically corresponding parts. The movements executed by the subjects may not always be symmetrical, and this asymmetry may be reflected in the difference in activity recorded from the two sides. Postural asymmetry is commonly found in the standing position, although the subject appears to be standing straight, and although he believes that he is in his most comfortable and relaxed attitude. This asymmetry may disappear when the muscles are thrown into action.

The earth connexion was usually made with an electrode placed approximately in the mid-line above the symphysis pubis.

RESULTS

Various movement patterns were studied with the subjects in the two postures (*a*) supine, and (*b*) standing. The subjects were told what movements to make but were not told how to make them. That is to say, they were left to make the movements in the manner that came naturally. No difference was recorded between the movement patterns in male and female subjects.

(*a*) *Supine posture*

The subject lay on a clinical examination couch, with the flap at the head end of the couch raised to an angle of approximately 30°. A moderate degree of comfort is essential if muscular relaxation is to be achieved, and to attain this a pillow was placed beneath the head and additional support given to the feet when they projected beyond the end of the couch.

(1) *At rest*

At rest, a slight discharge may be recorded from the obliques. The psychological state of the subject seemed to be reflected in the degree of resting discharge. Nervous subjects do not relax completely. Placid subjects, provided they are warm and comfortable, may achieve almost complete relaxation.

In those subjects showing a resting discharge, alteration of the angle of the flap of the couch can modify the discharge. Passive flexion of the trunk (by raising the flap) reduces the discharge: passive extension increases it.

(2) *Head raising*

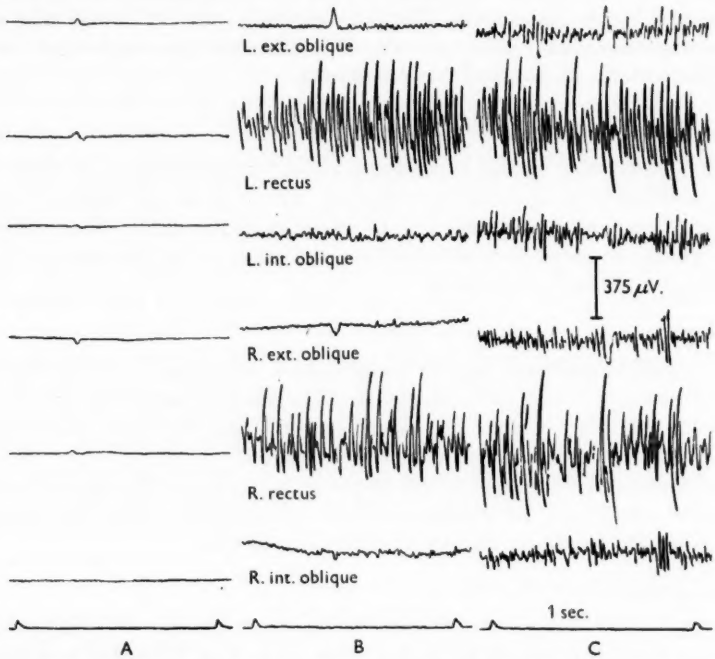
Text-fig. 1 shows the pattern of activity for the movement of head lifting, i.e. raising the head off the couch by flexion of the neck. This movement is carried out against gravity. The recti are mainly involved in this movement as shown by the large amplitude of the action potentials recorded from them as compared with those from the obliques.

In this movement the rectus may be regarded either as an agent in a general flexion movement, involving the neck and trunk, or as a fixator of the thorax. Text-fig. 2 shows that even when a slight effort is made, sufficient in this case to make the sterno-mastoids stand out but not sufficient to raise the head, the recti and sterno-mastoids contract almost simultaneously. The external oblique participates in this synergic movement. The retro-aponeurotic fibres of the internal oblique cannot be concerned immediately in the movement. However, these fibres may have a particular function in guarding the inguinal region. As shown later, in any movement which involves a rise of intra-abdominal pressure, these fibres contract and prevent bulging of the anterior abdominal wall in the inguinal region.

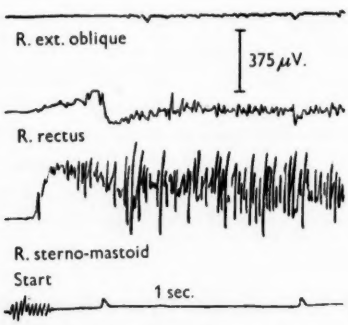
(3) *Straining*

Text-fig. 3 shows the pattern of activity for straining. The subjects were instructed to close the glottis and bear down. The external obliques contract. The activity recorded varies with the effort made. The internal oblique fibres show a parallel activity in guarding the inguinal region. The rectus abdominis, however, is involved

to a much less degree. Even when a maximal effort is made the rectus activity is much less than that recorded from the obliques, and less also than the activity recorded from the same rectus when the head is raised.



Text-fig. 1. Subject supine. Record A is control showing the resting state, with no action potentials recorded from the muscles. Record B shows muscle-action potentials demonstrating the pattern of activity for raising the head. Record C shows the action potentials when the effort is increased; the pattern of activity is similar. The e.c.g. can be seen, particularly in the external oblique tracings.



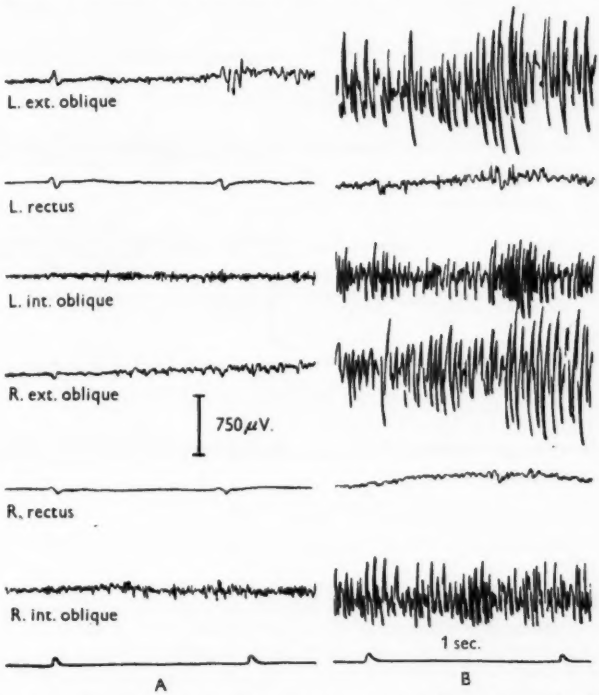
Text-fig. 2. Muscle-action potentials to show the synergic action of the sterno-mastoids and recti in the movement of raising the head from the supine position. The effort was minimal, sufficient to make the sterno-mastoids stand out, but not sufficient to lift the head.

(4) *Quiet breathing*

In the supine position the abdominal muscles show no increase of activity during expiration. Against this background reciprocal inhibition cannot be demonstrated during inspiration.

(5) *Forced expiration*

Text-fig. 4 shows the pattern of activity for forced expiration. The lungs were exhausted as far as possible. The pattern is similar to that demonstrated for straining,

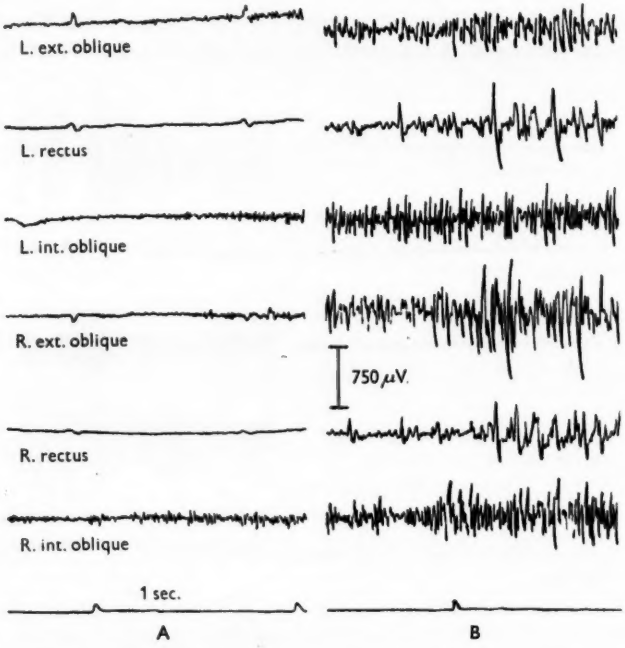


Text-fig. 3. Muscle-action potentials to show the pattern of activity in the supine position during straining. Record A shows the commencement of straining. No activity is seen in the recti, only the e.c.g. is visible. (The amplification is $\frac{1}{2}\times$ that used in Text-figs. 2 and 3.) Record B, 4 sec. later, shows the relatively slight involvement of the recti, as compared with the obliques, when a maximal effort was made.

but when a maximal effort is made there is a tendency to flex the trunk, to lift the head and to rotate the pelvis forwards, i.e. to go into a general attitude of flexion. If this happens then the basic pattern of activity for active expiration has added to it the pattern of activity for the flexion movement of the trunk. For this reason the rectus action was seen in this record. That this does actually occur was confirmed in experiments in which trunk flexion was deliberately carried out while a maximal expiration was made, and vice versa. So long as trunk flexion does not occur, the recti are not brought into action.

(6) *Coughing*

Text-fig. 5 shows the movement pattern for coughing. Coughing may be described as a brief 'strain'—glottis shut—while the intra-abdominal pressure builds up, followed by a short active expiration, i.e. an integration of straining and forced expiration. The relative inactivity of the rectus is apparent.



Text-fig. 4. Muscle-action potentials to show the pattern of activity in the supine position for a gradually increasing expiratory effort. Record A shows the commencement of the effort; there is minimal involvement of the recti. Record B, 3 sec. later, shows the pattern for a maximal effort. The involvement of the recti seen in record B is discussed in the text, and represents flexion of the trunk, superadded to the expiratory effort.

(7) *Singing*

Text-fig. 6 shows a graded effort from soft to loud-as-possible. The relative inactivity of the rectus is again apparent.

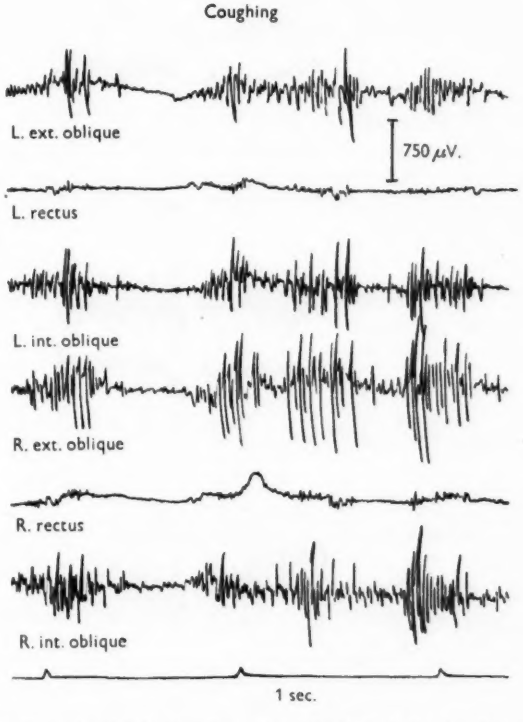
(8) *Leg raising*

When the legs are raised the pelvis is fixed by the trunk musculature, and all abdominal muscles contract (Text-fig. 7*a*). The most striking change between elevation of right leg alone and left leg alone is in the action of the ipsilateral internal oblique. Text-fig. 7*b* shows this.

(*b*) *The erect posture*

(1) *Resting discharge*

In order to show resting discharge high amplification was used. Text-fig. 8 shows that in the erect posture the internal oblique fibres are in a state of slight contraction. The contraction recorded from the external obliques and recti may be modified by movement backwards and forwards or from side to side, but the contraction of the internal oblique fibres is continuous. Their degree of resting tone normally

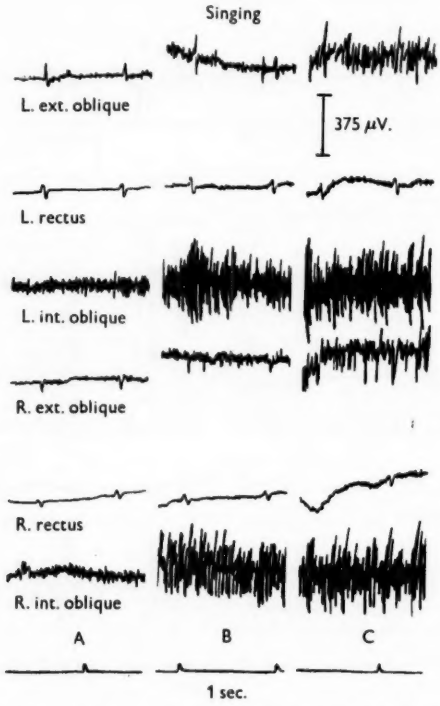


Text-fig. 5. Muscle-action potentials to show the pattern of activity in the standing position for coughing. The pattern for the supine position is similar. Note minimal involvement of the recti.

exceeds that of the other muscles of the abdominal wall. It is suggested that these fibres are continually on guard, and protect the inguinal region from rupture. When a straining effort is made, increasing the intra-abdominal pressure, the tone in these fibres increases.

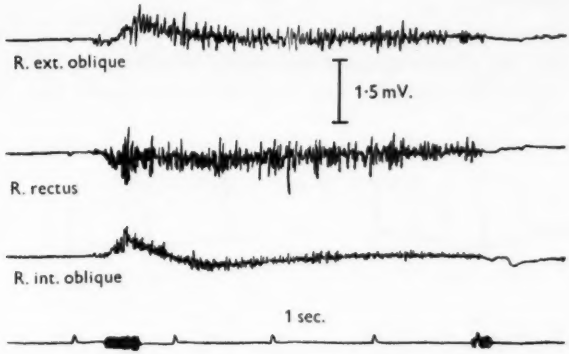
The action potentials picked up by the internal oblique electrodes do not come from the psoas. If the subject leans forwards with hips flexed at 30°, the hip flexors are relaxed, but the activity recorded from these electrodes continues unchanged (Text-fig. 9). If the subject leans backwards, the hip flexors contract to control the passive extension of the hips by gravity. If the internal oblique electrodes pick up

from the psoas, then the potentials recorded should increase when the subject leans backwards. In fact, they do not (Text-fig. 10). Further, with the subject supine, if the intra-abdominal pressure is raised to the maximum possible and if the right leg is then raised against gravity, no difference is recorded from the two pairs of internal oblique electrodes. Clearly if action potentials from the right psoas reach the right pair of internal oblique electrodes, then the record from the right side should exceed that from the left. In fact, it does not. These findings support the view that the electrodes record potentials from the anterior abdominal wall, and not from hip flexors.

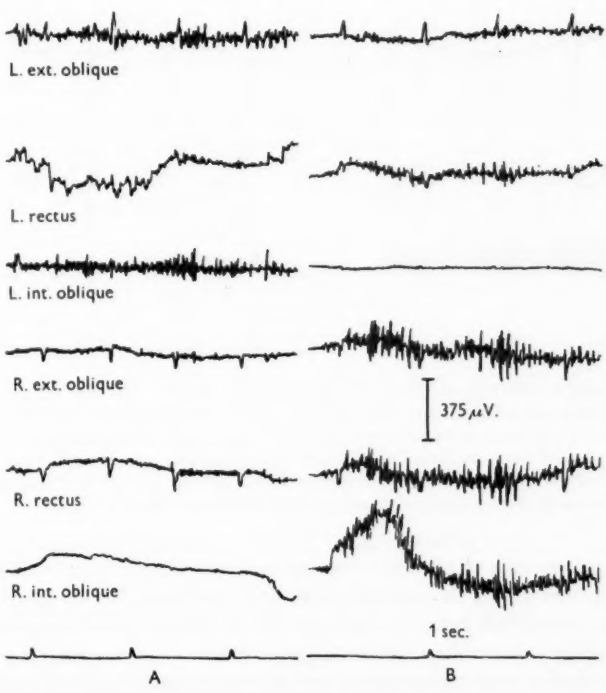


Text-fig. 6. Muscle-action potentials in supine position for singing. A, soft, B, louder, C, loud-as-possible. Note the similarity of pattern in A, B and C, and the minimal involvement of the recti.

The further possibility arises as to whether the cremaster contributes to internal oblique electrode pick-up. The contraction of the cremaster can be seen (in young adult males) to coincide with the voluntary contraction of the abdominal wall, e.g. in straining. The level of action potentials is unchanged during gentle elicitation of the cremasteric reflex, i.e. when the visible response is confined to the testicle. Strong elicitation results in a visible spread of the response to the abdominal wall with a corresponding increase in activity in the electrical record. Finally, the tonic contraction referred to in the erect posture is present in the female; thus the cremaster cannot be the sole origin of these action potentials.



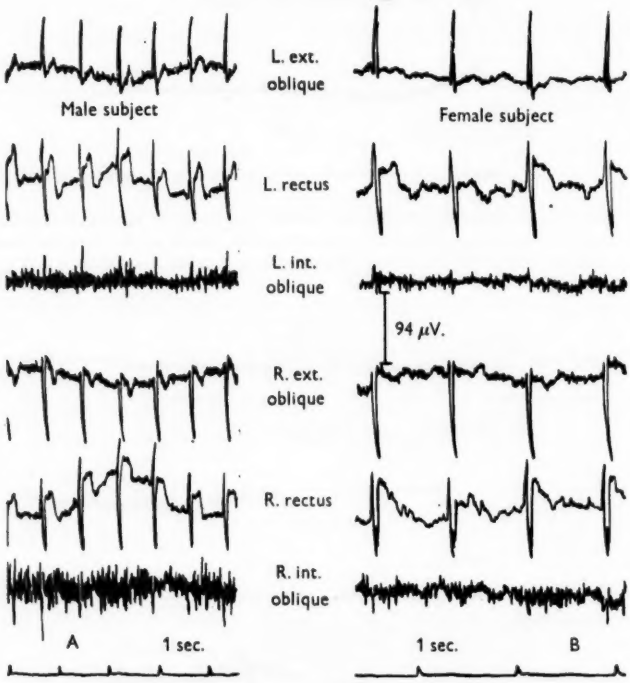
Text-fig. 7 (*a*). Muscle-action potentials to show the pattern of activity for raising the legs from the supine position. Both legs raised and fully extended, with heels about 12 in. above the horizontal. The muscles on the left side showed a similar pattern. (Note that the amplification is $\frac{1}{4}\times$ that used in Text-figs. 2 and 3.)



Text-fig. 7 (*b*). Muscle-action potentials to show the pattern of activity in the supine position for raising the legs. Record A shows left leg only raised, with the heel about 12 in. above the horizontal. Record B, raising right leg to the same extent. Note the relatively greater activity on the side of the movement in each record. This is particularly noticeable in the internal oblique.

(2) *Movement patterns*

(*a*) Those movements carried out in the recumbent position were repeated in the standing posture, viz. straining, quiet and forced breathing, coughing and singing. The patterns of activity observed did not differ significantly from those for the recumbent position.

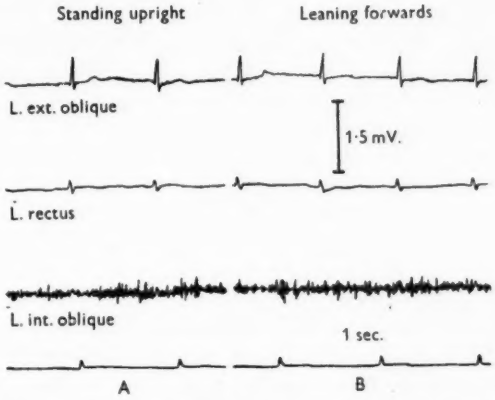


Text-fig. 8. Record A, male subject: Record B, female subject. Both records were taken at high amplification (4 × that of Text-figs. 2 and 3) to show the resting discharge in the standing position. Note the e.c.g. which appears in all leads, but does not obscure the muscle-action potentials which are clearly seen between each e.c.g. complex. The slow drift of the base-line, seen in some leads, is an artifact caused by movement of the abdominal wall. The chart speed was doubled for the right-hand record. Note the relatively greater activity recorded from the internal oblique electrodes.

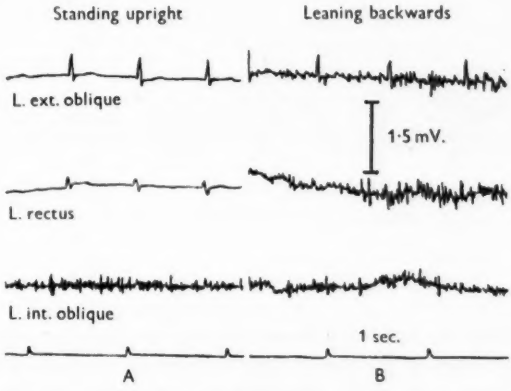
(*b*) Other movements studied were flexion and extension of the trunk, i.e. leaning forwards and backwards. In leaning backwards the trunk is extended by gravity. The erectores spinae are relaxed, and the effects of gravity are controlled by the flexors. Similarly, in leaning forwards, gravity flexes the trunk. The flexors are largely relaxed (the recti completely), and the erectores spinae contract. Clearly, as Duchenne (1867) and Beevor (1904) concluded, gravity is the prime mover in these movements.

(3) *Erectores spinae and straining*

When the intra-abdominal pressure is increased, the external oblique muscles will tend to flex the trunk. This may be regarded as an unwanted 'by-product' of raising the intra-abdominal pressure. To neutralize this 'side-action' the erectores spinae contract synergically. This contraction is graded, and parallels that of the external obliques.



Text-fig. 9. Muscle-action potentials from the left side to show the relative inactivity in external oblique and rectus, in the standing position, during the movement from: record A, standing upright, to record B, leaning forwards. Note the constancy of the internal oblique discharge. (See discussion in text re psoas muscle.)



Text-fig. 10. Muscle-action potentials from the left side to show the pattern of activity in the standing position during the movement from: record A, standing upright, to record B, leaning backwards. Note that the rectus and external oblique contract, and that the discharge from the internal oblique remains constant. (See discussion in text re psoas muscle.)

DISCUSSION

There is no instrumental or other simple way of summarizing quantitatively the amounts of activity found in different muscles when a whole series of movements is studied. The records obtained in these experiments are often 100 ft. long, and some of the movements are repeated many times. The consistency of the pattern of activity, for any one movement, has been judged by eye, and all the records were assessed for grades of activity in the different movements in this way on a 4-point scale (with due allowance for the different amplification employed with different subjects). In Table 1 are given the average grades of activity for the various movements. The grades used are: 0; +; ++; +++. Numbers were deliberately avoided because of the suggestion of measurement which they imply.

The records show that with 'head lifting' the rectus is primarily involved. This is well known and is often made use of in the clinical examination of the abdomen. The basic pattern for straining, however, is the antithesis of the 'head lifting' pattern and mainly involves muscles other than the rectus. In certain subjects the rectus seemed to be involved in straining. When these subjects are watched carefully, flexion movements of the trunk or pelvis are always seen to coincide with the rectus action, and the conclusion emerges quite clearly that straining alone does not involve the rectus. But if to the basic movement pattern for straining, flexion of the trunk is added, then evidently the flexor muscles must come into action, and the flexor pattern is superadded to the straining pattern.

Table 1. *Summary of the findings in all subjects for muscle-movement patterns studied*

(To construct the table each subject's record was examined, and assessed on the 4-point scale described in the text, i.e. 0, +, ++, +++. The Table gives, for each movement, the assessment averaged over all the subjects in whom the movement was studied. The figures in brackets give the number of different subjects in whom each particular movement was studied.)

	Supine					Standing upright	
	Head raising (18)	'Straining' (13)	Raising left leg (13)	Raising right leg (13)	Raising both legs (10)	'At ease' (16)	'Straining' (13)
L. ext. oblique	+	+++	+	+	+++	+	+++
L. rectus	+++	+	+	+	+++	0	+
L. int. oblique	+	+++	++	+	+++	++	+++
R. ext. oblique	+	+++	+	+	+++	+	+++
R. rectus	+++	+	+	+	+++	0	+
R. int. oblique	+	+++	+	++	+++	++	+++

This basic pattern for straining demonstrates the primary importance of the rectus sheath, and hence of the oblique and transverse muscles in maintaining the integrity of the rectus part of the anterior abdominal wall, and suggests that the rectus muscle itself occupies a secondary place only. The importance of careful repair to the incised rectus sheath, and the possible danger of plastic proceedings displacing flaps of the rectus sheath, are evident. The tension felt in this region during clinical examination, when the subject strains or coughs, is largely due to the rectus sheath rather than the rectus muscle.

Photograph to show the placing of electrodes and the belt of rubber tubing by which they are retained in position. The actual wiring of the electrodes is not shown.

SUMMARY

1. An electromyographic method is described for demonstrating the patterns of activity of the surface muscles of the anterior abdominal wall, as seen in fifteen males and six females, aged 18–25.

2. Three pairs of electrodes were placed on each side as follows: (*a*) over the recti, (*b*) over the external obliques in the flank, (*c*) over the retro-aponeurotic part of the internal obliques.

3. The pick-up from each pair of electrodes was fed simultaneously through a 6-channel amplifier (standard Ediswan electroencephalograph).

4. The movements studied were:

(*a*) *Supine posture*

Head raising. The dominant role of the rectus is shown.

Straining. The action of the external and internal obliques is shown. The rectus plays a passive role in straining, and is only involved when the trunk is flexed.

Similar patterns were shown for active expiration, coughing, and singing.

(*b*) *Standing posture*

The level of tone in the internal oblique fibres exceeded that recorded from the external obliques and recti. The relation of this observation to the functional anatomy of the inguinal region is discussed.

The patterns for straining, forced expiration, coughing and singing were shown to be similar to those obtained in the supine position. In addition the synergic contraction of the erectores spinae in straining was recorded.

Flexion and extension of the trunk demonstrated the reciprocating action of recti and erectores spinae.

We are indebted to Prof. John Kirk and Prof. Samson Wright for their encouragement and helpful criticism of this work and to Mr D. H. Patey for drawing our attention to the problem of the inguinal canal. We are also grateful to the University of London Central Research Fund for a grant to one of us (W.F.F.) for some of the equipment used in this research. Finally, it is a pleasure to acknowledge the friendly co-operation of our students, and the technical assistance of Miss D. Anderson and Mr W. Cheeseman.

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AN EXPERIMENTAL STUDY OF THE VISUAL PATHWAYS IN A REPTILE (*LACERTA VIVIPARA*)

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The structural analysis of the reptilian brain has so far been based almost exclusively on the study of normal material prepared by various neuro-histological techniques. By such means accurate and detailed maps of the disposition of cells and fibre tracts have been made. I refer, in particular, to the studies of Huber & Crosby on *Alligator* (1926) and on *Anolis* (1933), of Cairney (1926) and Durward (1930) on *Sphenodon*, and of Shanklin (1930) on *Chameleon*. Though these form a base on which further investigations can be built, they do not by themselves provide adequate or satisfactory material for morphological and functional interpretation. They give little objective evidence of the precise origin or termination of fibres, or of the direction in which they normally conduct. To gain this information it has been found necessary to make use of methods for the differential staining of degenerating fibres; but such methods have been used extensively only on the mammalian brain.

It is probably in the analysis of the mammalian visual system that the use of degeneration techniques has met with most success. It was therefore decided to apply these methods to that system in the reptile, in order to determine the course followed by fibres of retinal origin through the chiasma and optic tracts, and also the localization of their terminals in the brain. In addition, a large part of the investigation was necessarily devoted to a study of the nature and rate of degenerative changes in the reptilian nervous system.

The methods available fall into three classes, depending on the demonstration of degeneration in: (*a*) the cell body of the neuron; (*b*) the axon and its terminals; and (*c*) the myelin sheath. The Marchi technique for the demonstration of myelin degeneration has been used, but is not very satisfactory in the reptilian brain, where many fibres are unmyelinated; chief reliance has therefore been placed on the demonstration of the degeneration of axons and their terminals by means of a silver impregnation technique. No attempt has yet been made to utilize degenerative changes in the cell body, assuming these to occur in reptiles as they do in mammals.

MATERIAL AND METHODS

The animals used were specimens of *Lacerta vivipara*. Some were prepared as normal material; in others the left eye was removed under ether anaesthesia, and penicillin-sulphathiazole powder introduced into the orbital cavity. The mortality rate was high, one in three of the animals dying as the immediate result of the operation. The post-operative behaviour of the animals was apparently normal, and infection occurred in only one specimen. In some preliminary experiments, in which snakes

* This work was carried out during the tenure of a post-graduate studentship from the University of London.

as well as lizards were used, the animals were kept at room temperature (approximately 16° C.). Under those conditions no degenerative changes could be detected even 17 days after the operation. In all subsequent experiments, therefore, the lizards were kept in cages heated to approximately 30° C. At intervals between 6 days and 11 weeks after the operation the animals were killed by decapitation. The brain was exposed and fixed *in situ* for 24 hr. prior to removal.

Histological methods

(*a*) Five Nissl series were prepared from normal specimens. The brain was fixed either in 95 % alcohol or in 12 % formol saline with 4 % acetic acid. After alcohol fixation three transverse series were stained with thionin, and after formol-acetic fixation one transverse and one horizontal series were stained in the same way. The sections were all cut in paraffin at 12 μ.

(*b*) In preliminary trials the most complete and consistent silver impregnations were obtained by Nonidez' modification (1939) of the Cajal technique. This method was therefore used throughout, except for two brains prepared by the pyridine-silver method which also gave satisfactory results.

The normal silver material consisted of five brains impregnated by Nonidez' method, of which two were cut transversely and three horizontally. The experimental material included eighteen brains treated by Nonidez' method, of which sixteen were cut transversely and two sagittally. Of the transverse series two were prepared 6 days after removal of the eye, and the remainder 7, 8, 10, 12, 13, 14, 17, 20, 25, 26, 34, 36, 69 and 77 days after. Of the sagittal series one was prepared after 13 days, the other after 15 days. In addition, two transverse series were prepared by the pyridine-silver method 15 and 23 days after the operation. All the silver material was cut at 7 μ.

(*c*) One normal transverse series was stained by the Weigert-Pal method. Two experimental series were prepared by the Marchi technique, 25 and 26 days after the operation respectively. Both were cut transversely.

The normal anatomy of certain thalamic and mid-brain centres

It appears from the literature that between different species of reptiles there exists considerable variation in the degree of development of certain nuclei, and that normal histological appearances have been variously interpreted. It is therefore necessary to describe, in some detail, the structures in *Lacerta* to which particular terms are to be applied. So far as possible, common usage has been followed in the terminology adopted, but its use does not necessarily imply homology with structures similarly named outside the reptilian class.

The diencephalic region

Lateral geniculate nucleus. This is a well-defined formation situated in the lateral part of the thalamus. The whole nucleus is divisible into medial and lateral parts, which I have called the 'medial cell plate' and the 'lateral neuropil' (Text-fig. 1*b*). The latter is an area of dense neuropil related directly to the concave surface of the optic tract. Small, rounded cells are scattered throughout it. Its dorsal extremity

lies lateral to the conspicuous nucleus rotundus, while ventrally it extends to the lateral aspect of the lateral forebrain bundle. The medial part of the nucleus is a vertical plate of fairly large, fusiform cells. It extends not quite as far dorsally as the lateral neuropil, and its ventral border is formed by a segregated cluster of fusiform cells dorsolateral to the lateral forebrain bundle. Each cell lies with its long axis approximately perpendicular to the optic tract, and from the cell body processes extend both laterally and medially. Those directed laterally are coarse; they enter and ramify within the lateral neuropil, many of their branches reaching up to the optic tract. Those directed medially are fine; they turn ventrocaudally and pass to the tegmentum in a large tract, the fasciculus geniculatus descendens (Text-fig. 3). Although the present material does not reveal the precise termination of this tract, it is seen to converge upon the retro-infundibular decussation, within which it may cross.

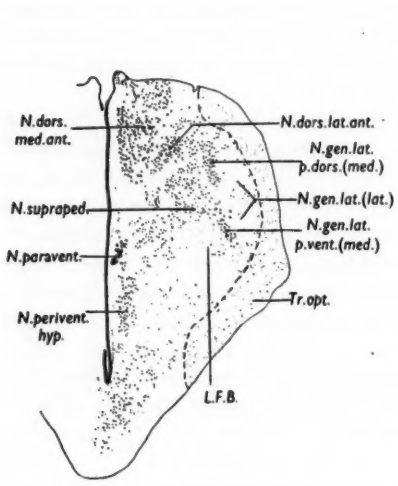


Fig. 1*a*.

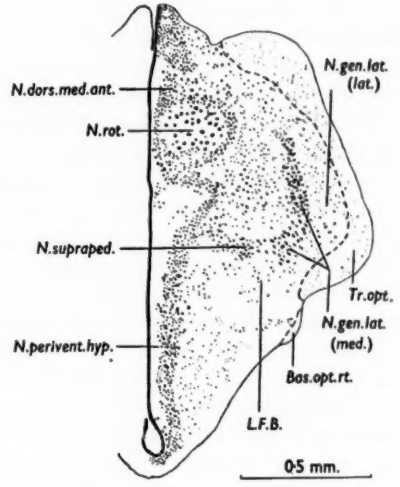


Fig. 1*b*.

Text-fig. 1. Scale drawings of transverse sections stained with thionin: (*a*) through the rostral part of the thalamus; (*b*) through the middle of the thalamus. For key to abbreviations see p. 166.

The extreme rostral end of the geniculate nucleus separates into dorsal and ventral parts (Text-fig. 1*a*). The pars ventralis is closely related to the lateral forebrain bundle, and the fusiform cells forming its medial part are a forward extension of the cell cluster which at more caudal levels forms the ventral border of the medial cell plate. In the pars dorsalis, however, the medial plate cells become small and rounded, and do not appear to give rise to fibres of the fasciculus geniculatus descendens. They are not clearly distinguishable from the cells of the nucleus dorsolateralis anterior of the thalamus. The lateral neuropil of the pars dorsalis is less dense than in other regions of the nucleus.

Comparable descriptions of the lateral geniculate nucleus were given by Edinger (1899) for *Varanus*, and by Beccari (1923) for *Lacerta muralis*, the latter referring to the medial cell plate as the 'cellule a doppio pennacchio'. Elsewhere little stress

has been laid upon a differentiation into medial and lateral parts. In many reptiles the nucleus appears to be less well developed than in *Lacerta*, but it is clear that some authors have regarded the lateral neuropil as the whole nucleus. This is certainly true of Frederikse (1931), who refers to the medial cell plate in *Lacerta* as a separate 'nucleus lateralis'.

Dorsoventral subdivision of the geniculate nucleus has been described in various reptiles, e.g. by Ramón (1896) and by Shanklin (1930) in *Chameleon*, by Cairney (1926) and by Durward (1930) in *Sphenodon*, by Huber & Crosby (1933) in *Anolis*, and by Warner (1947) in *Crotalus*. The subdivisions have varied considerably between the different species with regard to both the number and character of the separate parts so formed. In the present study of *Lacerta* definite separation into a dorsal and a ventral part could be distinguished only in the rostral part of the nucleus where, as described, the pars dorsalis presents certain features in which it differs from the rest of the nucleus. It is true that the segregation of a cluster of cells along the ventral border of the medial cell plate also suggests a division of the nucleus at more caudal levels. But such a division is not justified by any difference in either cellular morphology or connexions, nor by the results of the experimental investigation. Cairney (1926) and Durward (1930) found similar conditions in *Sphenodon*, where separate dorsal and ventral parts of the nucleus could be identified only in the rostral thalamus. It is possible that the pars dorsalis described here in *Lacerta* may correspond to a lateral differentiation of the nucleus dorsolateralis anterior which Huber & Crosby (1926) described in the alligator.

Pretectal region

Beneath the rostral part of the optic tectum, in a zone of transition from thalamus to midbrain, is the pretectal region. It contains the following nuclei which are of significance for the present investigation.

Nucleus lentiformis mesencephali. This is a collection of large and small cells extending obliquely across the base of the rostral border of the tectum (Text-figs. 2*a*, and 3). Its outline is not well defined. The larger cells are fusiform, and lie in the rostral part of the nucleus in relation to optic tract fibres ascending to the anterior aspect of the tectum. The nucleus is pervaded by a transverse band of fibres, the brachium tecti medialis (Shanklin, 1930; Huber & Crosby, 1933). It is succeeded caudally by the nucleus pretectalis.

Nucleus pretectalis. This is a clearly circumscribed structure containing closely packed cells of medium size and a fine neuropil. In transverse sections it appears as an ovoid body lying at the level of the posterior commissure, caudal and slightly ventral to the nucleus lentiformis. In sagittal sections its shape is round (Text-fig. 3). Dorsally it is related to the stratum griseum centrale of the optic tectum (vide infra) and it is traversed by bundles of fibres passing to, or from, the deeper layers of the tectum.

Nucleus geniculatus pretectalis (Text-fig. 2*a*). This is situated ventral to both of the above-mentioned nuclei. Its rostral end lies dorsomedial to the caudal extremity of the lateral geniculate nucleus to which it is very similar in structure, but from which it is clearly separated. Its medial part consists of quite large fusiform cells,

and the lateral part is a region of dense neuropil related to the inner surface of the optic tract. Its extent is appreciated only after combined study of Nissl and silver preparations. Coarse dendritic processes extend laterally from the fusiform cells into the neuropil. Finer processes are directed medially into a large tract, the fasciculus geniculatus pretectalis descendens. This tract runs ventrocaudally into the tegmentum, in company with the fasciculus geniculatus descendens (Text-fig. 3).

The nucleus posterodorsalis. In a transverse series this first appears lateral to the habenula. It contains a few small cells and a diffuse neuropil. Caudally it extends above the posterior commissure as a rounded elevation medial to the optic tectum and close to the midline (Text-fig. 2*a*). It is penetrated by fibres of the tecto-thalamic system, and in normal preparations appears to be reached by fibres from the supra-optic decussations and from the optic tract.

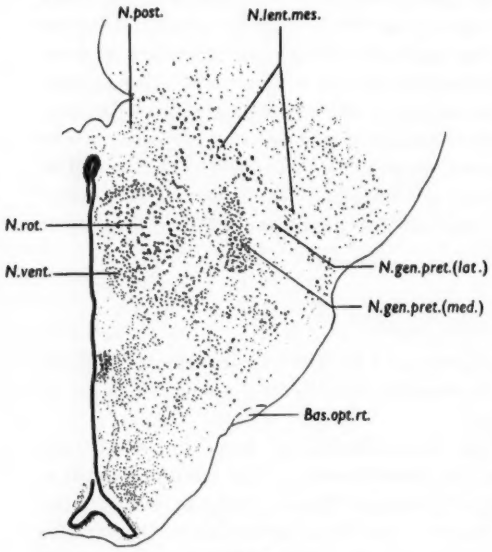


Fig. 2*a*.

Fig. 2*b*.

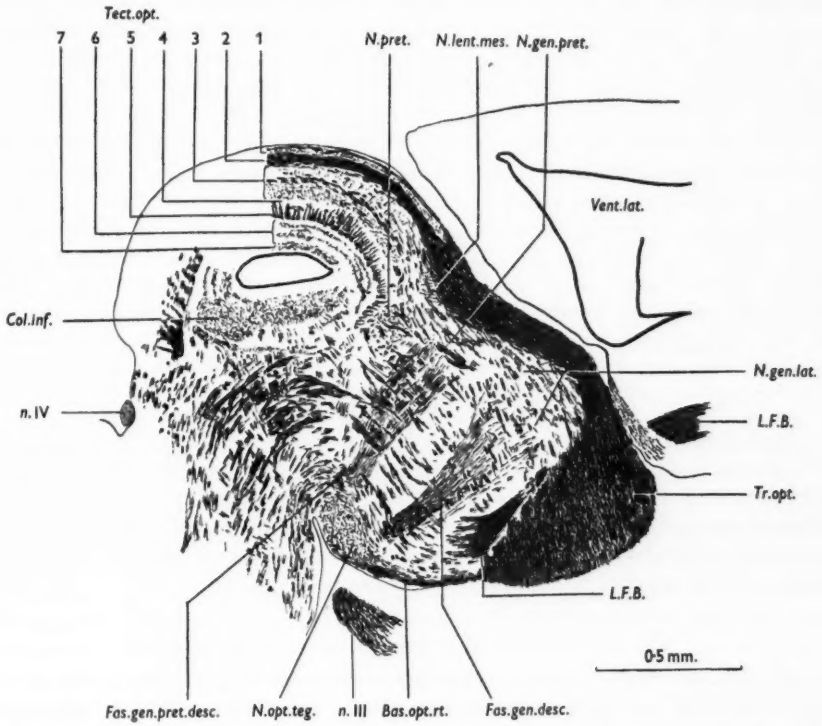
Text-fig. 2. Scale drawings of transverse sections stained with thionin: (*a*) through the pretectal region; (*b*) through the midbrain. For key to abbreviations see p. 166.

The present description cannot easily be reconciled with all previous accounts of the pretectal region in reptiles. It is very similar to that of Beccari (1923) for *L. muralis*, and is comparable with those of Huber & Crosby (1926, 1933) and Cairney (1926). There is no doubt that much confusion has been caused simply by terminological inconsistencies. Thus, the 'nucleus lentiformis' described by Edinger (1899) in *Varanus* is the nucleus pretectalis of many of the more recent workers, while his 'nucleus pretectalis' is probably the nucleus geniculatus pretectalis. Edinger's terminology was adopted by de Lange (1913). The nucleus posterodorsalis of the present work corresponds to the structure of the same name described by Huber & Crosby in *Anolis* (1933) which was later identified by Papez (1935) in turtles as the

'area pretectalis'. Frederikse (1931) failed to distinguish in *Lacerta vivipara* the nucleus geniculatus pretectalis, and much of his interpretation of the pretectal region is not confirmed by the present material.

Optic tectum (superior colliculus)

The optic tectum of *Lacerta* has a complex laminar structure in which seven principal strata can be recognized (Text-figs. 2*b*, 3). Of these, only the first three



Text-fig. 3. Scale drawing of parasagittal section through the diencephalon and midbrain. (Nonidez' silver method.) For key to abbreviations see p. 166.

are of concern to the present investigation. They are, from the surface inwards, as follows:

Stratum zonale (layer 1 in the figures). This is present as a thin layer over the entire surface of the tectum, but is much thicker on the anterior, medial and dorsal aspects. It consists of a neuropil of fine fibres with scattered small cells. Over approximately its rostral half it is characterized by the presence of small fibre bundles just beneath the pia mater; they appear to enter the stratum from the optic tract. Huber & Crosby (1933) indicate that the stratum zonale is poorly developed in many reptiles, but in *Lacerta* and probably in *Sphenodon* (Cairney, 1926) it is a well-defined structure.

Stratum opticum (layer 2 in the figures). This is a dense layer of closely packed fibre bundles coming from the optic tract. Small cells are scattered between them.

Stratum fibrosum et griseum superficiale (layer 3 in the figures). A broad layer which in silver preparations is readily divisible into four substrata on the basis of differences in the density of the neuropil and the relative number of fibre bundles. Cells are scattered throughout, but apart from a slight concentration in the second substratum the lamination observed in the Nonidez preparations is not evident in the Nissl material. The outer substratum is distinguishable from the overlying stratum opticum only by the presence of areas of coarse neuropil between the bundles of fibres. The second substratum is a broad zone of diffuse, fine neuropil with only a few fibre bundles; the third is narrow, and like the first consists of a coarse neuropil and many fibre bundles; the fourth has a dense, fine neuropil and only a few fibre bundles. The fibre bundles referred to are arranged tangentially, and are derived from a number of sources, including the optic tract, the supraoptic decussations and the so-called thalam-otectal pathways. Traversing the whole stratum fibrosum et griseum superficiale there are, in addition, many radially directed fibres. The origin and termination of these could not be determined on account of the density of the fibre plexus, but they probably include ascending processes from cells lying in the deeper strata of the tectum, including the periventricular layers. de Lange (1913) and Huber & Crosby (1933), using Golgi preparations, showed such fibres to exist in the reptilian tectum.

EXPERIMENTAL RESULTS

In experimental material prepared by silver impregnation degenerative changes were revealed in the fibres of retinal origin. This provided a positive means of identification, and permitted a more precise analysis of the visual pathways than is possible with normal material. As very little is known about degeneration in the reptilian nervous system it is necessary to describe in some detail the histological characteristics of the degeneration observed in the present experiments.

The characteristics of axonal and terminal degeneration

In specimens prepared 6 days after the operation no change could be detected in axons from the cut optic nerve. In the 7-day specimen they showed a distinctly heavier impregnation, and this increased affinity for silver remained the only indication of degeneration up to 10 days. After 12 days definite changes were evident in axonal structure, and were well established by the 15th day (Pl. 1, fig. 1). Many fibres had lost their smooth contour, becoming sinuous and uneven. Irregular varicosities had developed along the course of the axons, some of which presented very large spherical and ovoid swellings separated by constrictions which appeared to be on the point of rupture. Large irregular vacuities were present in the midst of degenerating fibre bundles, and became an increasingly prominent feature as degeneration proceeded. After 25 days fragmentation of the distorted axons was well advanced. This resulted not only in the formation of many short lengths of tortuous and heavily staining axons (Pl. 1, fig. 2), but also gave rise to a wealth of small, irregular fragments and of large fusiform and spherical bodies along the course of

the visual pathways. By 36 days the original fibre bundles were largely replaced by a dense mass of axon debris and a lightly staining amorphous ground substance. Resorption of the debris appeared to be a slow process, and was far from complete even after 11 weeks.

Degeneration of most fibres followed the sequence described, but certain variations suggest that the rate of degeneration is related to axonal thickness. Unusually coarse axons, such as those of the basal optic root, disintegrated rapidly into blackened granular fragments (Pl. 1, fig. 3*b*), this being already evident after 12 days. On the other hand, structural changes in many of the finest axons and collaterals were not apparent until after 34 days.

In addition to axonal degeneration changes were observed which appear to indicate the terminal distribution of degenerating fibres. These changes were very similar to the mammalian terminal degeneration first described by Hoff (1932), but with one outstanding difference. Mammalian studies have been concerned with the degeneration of terminal boutons (end feet) which are a normal feature of many synaptic junctions. With the technique employed here it was not found possible to demonstrate the existence of boutons in the normal lizard material in any of those parts of the brain related to the fibres of retinal origin; but 6 days after removal of the eye a profusion of minute argentophil rings was demonstrable in some of these regions. Although some of the rings appeared to be isolated structures many were clearly terminal formations, each on the end of a short length of fine fibre (Pl. 2, fig. 4). The point of attachment to the fibre was usually drawn out to form a racquet-shaped outline. The precise disposition of the rings varied, depending upon the particular locality in which they were observed. Sometimes they lay singly or in clusters in relation to cell bodies, but more frequently in relation to dendritic processes. In all of the areas concerned some also appeared in the sections to lie freely in the neuropil, not obviously related to either cells or fibres. The histological appearance of many of these structures was the same as that of the normal terminal boutons as described, for example, in the lateral geniculate body of mammals (Glees & Le Gros Clark, 1941; Glees, 1941, 1942), but their appearance in the lizard must be regarded as a degenerative phenomenon since they were demonstrable only after removal of the eye.

Between 12 and 15 days after the operation the rings were larger and much more numerous, some having become markedly elongated, and the majority showed varying degrees of thickening with consequent reduction of the lumen. In some the lumen appeared to have been entirely obliterated, resulting in the formation of solid bulbs some of which were almost as large as the nucleus in nearby cells (Pl. 2, figs. 6, 8). By this time many fine fibres of the surrounding neuropil showed swelling, vacuolation and fragmentation. This was often seen in fibres bearing a terminal ring or bulb, and a characteristic thickening of the fibres was frequently observed where they divided (Pl. 2, fig. 10*a*). Fibre changes of this kind have been referred to in mammals as 'preterminal degeneration' (Le Gros Clark & Meyer, 1947).

On the 20th day the number of rings appeared to be less, but the proportion of bulbs was increased. Many of the latter were very large, and some showed signs of granular disintegration. At 25 days only a few remained, and there was a noticeable sparcity of neuropil in the regions concerned. Nevertheless, both rings and bulbs

were occasionally found even after 36 days, some being very small and no different in appearance from those first seen at 6 days. None remained 10 weeks after the operation.

The visual pathways

In the specimen prepared 11 weeks after the operation no normal fibres could be found in the debris in the central stump of the divided optic nerve, which must therefore contain only afferent fibres. On reaching the chiasma the nerve divides into a number of large bundles which interdigitate with those from the opposite side, presenting in transverse section the dovetail pattern usual in reptiles. The number, size and arrangement of the bundles are very variable. In the present material anything from three to seven bundles were formed in different specimens, and further subdivision within the chiasma was occasionally encountered.

The bundles rejoin after crossing and form the greater part of the contralateral optic tract. Decussation, however, is not quite complete. A small fascicle separates from the most ventral bundle derived from the optic nerve and turns on to the ventral surface of the homolateral optic tract. Degeneration of this fascicle was initially overlooked on account of its small size, and it was first observed in a slightly oblique transverse series in which it was particularly obvious (Pl. 3, fig. 11). Re-examination of the other experimental material confirmed its existence, and in each case a corresponding fascicle of normal fibres was seen to turn on to the ventral aspect of the degenerating optic tract.

While recognizing the existence of a partial decussation, it must also be emphasized that the proportion of uncrossed fibres is in fact very small. Nevertheless, their presence in a reptile was an unexpected finding, and it is therefore appropriate to record here that similar experiments now in progress on the grass snake (*Natrix natrix*) have revealed uncrossed fibres in that species also (Pl. 4, fig. 13).

The degeneration of retinal fibres in the chiasma provided a clear demonstration of the extensive supraoptic system, which was of course totally unaffected by removal of the eye (Pl. 4, fig. 14). It is divisible into the usual dorsal and ventral supraoptic decussations which, as in reptiles generally, lie almost entirely caudal to the chiasma. The division itself is somewhat arbitrary, since there is appreciable interchange of fibres between the two decussations.

The ventral supraoptic decussation consists of a large band of fine fibres situated immediately caudal to the chiasma, from which it is not easily distinguishable in normal preparations. It appears to have extensive connexions with the hypothalamus. When followed laterally its fibres gradually separate into dorsal and ventral groups, which differ in their distribution and correspond to the dorsal and ventral parts of the ventral supraoptic decussation as described in *Sphenodon* by Cairney (1926). In their subsequent course the fibres of the pars ventralis form a compact bundle along the caudal margin of the optic tract. Those of the pars dorsalis swing on to the medial surface of the optic tract; further reference is made to them later.

The dorsal supraoptic decussation is smaller than the ventral. Its fibres cross directly beneath the third ventricle and spread out dorsolaterally, many of them penetrating the basal forebrain bundle. Coarse fibres situated in the caudal part of this decussation represent the 'fibrae ansulatae' of earlier writers.

The optic tract presents the usual gross relationships. It runs dorsolaterally from the chiasma on the ventral surface of the diencephalon. Turning round the lateral forebrain bundle it then passes dorsocaudally on the lateral surface of the thalamus towards the tectum of the midbrain.

Enucleation of the eye resulted in massive degeneration in the contralateral optic tract. Close to the chiasma a few degenerating fibres were seen to separate from its dorsal surface, to pass for a short distance through the supraoptic region of the hypothalamus, and then to rejoin the tract. No evidence was found for the termination of retinal fibres in any part of the hypothalamus.

Just lateral to the chiasma some remarkably coarse fibres become segregated in the caudal part of the optic tract. On nearing the lateral forebrain bundle they separate from the tract and run directly caudally as a compact bundle on the ventral surface of the subthalamus and tegmentum. This is the basal optic root or 'posterior accessory optic tract' (Text-fig. 3). Slightly rostral to the exit of the oculomotor nerve it enters the ventral aspect of the nucleus opticus tegmenti. The latter is a well-defined structure forming a pear-shaped elevation on the ventral surface of the tegmentum. It contains a dense, coarse neuropil and many multipolar cells of medium size (Text-figs. 2*b*, 3). Removal of the left eye resulted in complete degeneration of the contralateral basal optic root (Pl. 1, figs. 3*a*, *b*), and a conspicuous terminal reaction occurred throughout the corresponding nucleus opticus tegmenti. Argentophil rings appeared in relation to the cell bodies and their processes, and after 13 days many of them had developed into massive, heavily staining terminal bulbs (Pl. 2, fig. 8). Several such bulbs were frequently applied to the surface of a single cell body. After 11 weeks the nucleus was markedly shrunken as compared with the normal side, and much of its neuropil had disappeared.

On the lateral surface of the thalamus the optic tract is related medially to the lateral geniculate nucleus, and corresponds to the marginal optic tract of Huber & Crosby (1926, 1933). Degeneration experiments showed that the lateral neuropil of the geniculate nucleus receives a very large number of retinal fibres which turn medially from the tract, some traversing the width of the tract in order to gain the geniculate neuropil. Some are stem fibres, but many are collaterals which arise at right angles from fibres continuing dorsally in the tract.

From the 6th day after removal of the eye a multitude of argentophil rings was present in the contralateral geniculate nucleus (Pl. 2, fig. 4). They were very numerous in the lateral neuropil, being particularly dense close to the optic tract. Many were obviously related to the dendritic processes arising from fusiform cells of the medial cell plate, and others occurred in clusters around some of the small cells which are present in the lateral neuropil (Pl. 2, fig. 5). Rostrally, where dorsal and ventral subdivisions of the geniculate nucleus can be recognized, retinal fibres were traced into both subdivisions but especially into the ventral, where an exceptionally dense terminal reaction was obtained.

In the medial cell plate the terminal reaction was much less striking than in the lateral neuropil, although solitary rings were seen in relation to many of the fusiform cell bodies.

From the 12th to the 20th day after the operation the number of rings in the geniculate nucleus was much increased, and many terminal and isolated bulbs were

now present (Pl. 2, fig. 6). There was also abundant swelling, vacuolation and fragmentation of fibres in the lateral neuropil. After 11 weeks much of the neuropil had disappeared, and together with shrinkage of the optic tract this resulted in pronounced asymmetry of the diencephalon.

The boundary between the optic tract and the lateral geniculate nucleus is not easily defined, for some fibre bundles situated deeply in the tract encroach upon the outer part of the lateral neuropil. In sections through the rostral part of the thalamus a few such bundles of degenerating fibres were followed more deeply into the lateral neuropil of both the dorsal and ventral parts of the geniculate nucleus. Most of them appeared to terminate here, and the remainder rejoined the inner surface of the optic tract. These deeper bundles correspond, in part, to the 'axillary optic tract' of Huber & Crosby (1926, 1933). In the rostral pole of the thalamus a few retinal fibres were followed into a small, ovoid group of closely packed cells, the form and relations of which show that it corresponds to the nucleus ovalis of other reptiles (Huber & Crosby, 1926; Cairney, 1926; Durward, 1930; Addens, 1938). All appeared to be fibres of passage, and no evidence was found for the termination of retinal fibres in the nucleus ovalis.

Beneath the rostral part of the tectum the optic tract is in close relation to the nuclei of the pretectal region. Degenerative changes showed that many retinal fibres, including both stem fibres and collaterals, here turn medially from the tract and pass dorsal to the caudal end of the lateral geniculate nucleus. They enter the nucleus geniculatus pretectalis and the nucleus lentiformis mesencephali.

Six days after enucleation of the eye a profusion of argentophil rings was evident in the lateral neuropil of the contralateral nucleus geniculatus pretectalis. These were accompanied at 12 days by numerous isolated and terminal bulbs, and by the usual preterminal changes in many fibres of the neuropil. In this nucleus, as in the lateral geniculate, the lateral neuropil appears to be essentially an area of axo-dendritic association between incoming retinal fibres and the dendrites of the fusiform cells in the medial part of the nucleus. Only a relatively small number of rings and bulbs appeared actually in relation to the fusiform cell bodies.

The retinal fibres which enter the nucleus lentiformis mesencephali pass dorsal to the nucleus geniculatus pretectalis. They form a part of the so-called brachium tecti medialis, although much of this remained unaltered 11 weeks after removal of the opposite eye. The nucleus also receives retinal fibres which leave the optic tract as it ascends on to the anterior aspect of the tectum. An appreciable terminal reaction occurred throughout the nucleus lentiformis, but was less dense than that in the lateral geniculate nucleus and in the nucleus geniculatus pretectalis. In the rostral part of the nucleus clusters of bulbs and rings lay in relation to some of the larger cells and their processes (Pl. 2, fig. 7), but for the most part they were scattered freely in the neuropil. After 36 days much of the neuropil had vanished, its place being taken by irregular vacuities.

Degenerating fibres could not be followed into the nucleus pretectalis, nor into the nucleus posterodorsalis. In the sagittal series prepared 13 days after the operation several rings and bulbs were found amongst the cells of the contralateral nucleus pretectalis, but as this was not found in other specimens, its significance is questionable.

As the optic tract was followed dorsally in the experimental preparations it was

evident that it contained, particularly in its deeper part, an increasingly large number of fibres which were unaffected by removal of the opposite eye. This was especially clear in material prepared 10 and 11 weeks after the operation, in which crossed retinal fibres had undergone complete degeneration.

The small fascicle of uncrossed retinal fibres accounted for only a very small fraction of the persisting normal fibres. Its course and distribution are considered later, so that here we are concerned with non-retinal components of the optic tract. Most of these come from the ventral supraoptic decussation. Fibres which constitute the pars dorsalis of this decussation swing on to the medial aspect of the optic tract, and pass between it and the lateral forebrain bundle (Pl. 4, fig. 14). Here a few of them enter the tract, and accompany the fibres of retinal origin round the thalamus. The majority, however, form small bundles which sweep dorsocaudally through the lateral part of the thalamus and pretectal region. Many of these bundles incline laterally and join the inner surface of the optic tract, within which they continue dorsally towards the tectum. A relatively small number of fibres separate from the pars ventralis of the ventral supraoptic decussation, and enter the optic tract along its caudal margin.

After complete degeneration of crossed retinal fibres there also remained in the optic tract many normal axons which seemed to have no relation to the supraoptic decussations. They appeared to enter the tract from the lateral geniculate nucleus. Turning dorsally, usually at a right angle, they became mingled with the supraoptic fibres from which they could not then be distinguished. Such fibres were found in relation to all parts of the lateral geniculate nucleus, but the present material does not show whether they arise from or terminate within this nucleus.

When the optic tract arrives at the lower border of the tectum it has already contributed a considerable number of retinal fibres to the visual centres of the tegmentum, thalamus and pretectal region. In spite of this the majority of fibres from the retina eventually reach the tectum of the opposite side. It is likely that many of these have given collateral branches to the various diencephalic and pretectal centres. This was observed to be so in the case of the pretectal region, but from the available preparations it was not possible to be certain that collaterals which enter the lateral geniculate nucleus do indeed arise from fibres which ultimately reach the tectum.

On nearing the tectum the optic tract expands to form a broad sheet of fibres, those from the rostral part of the tract reaching the anterior and medial aspects, while those from the caudal part reach the lateral and posterior aspects. Degenerative changes showed that the great majority of retinal fibres pass from the optic tract into the stratum opticum, but that rostrally a few pass directly into the stratum zonale and also into the stratum fibrosum et griseum superficiale. The stratum opticum suffered almost complete degeneration after removal of the opposite eye. Over the whole extent of the tectum degenerating fibres were seen to leave the stratum opticum and enter the underlying stratum fibrosum et griseum superficiale, within which they ramified in the three outer substrata. The fibre bundles which pass directly into the stratum zonale form a narrow superficial layer of fibres (Text-fig. 3), and this degenerated completely. The few bundles which pass directly into the stratum fibrosum et griseum superficiale are mingled with many other bundles from the deeper part of the optic tract which did not degenerate and are thus not of

retinal origin. The latter include those supraoptic fibres which join the optic tract at thalamic and pretectal levels. No evidence was found to show that retinal fibres cross the midline in the tectum.

The most conspicuous terminal reaction within the tectum was seen in the stratum zonale, especially in its rostral part. Terminal rings and bulbs were distributed throughout the neuropil of this layer, and were associated with considerable varicosity and fragmentation of fibres of the neuropil itself (Pl. 2, figs. 10*a*, *b*). In the stratum opticum a few rings and bulbs were scattered sparsely between the fibre bundles, and in the stratum fibrosum et griseum superficiale they appeared in appreciable numbers in the three outer substrata (Pl. 2, fig. 9). Although such a very large number of retinal fibres enters the tectum it was surprising to observe that terminal changes never approached in density those which occurred in the other visual centres. From the twelfth day after the operation the most obvious feature of degeneration in the contralateral optic tectum was the disappearance of neuropil, especially from the stratum zonale and from the second substratum of the stratum fibrosum et griseum superficiale.

This description has been concerned so far with the distribution of retinal fibres which cross at the chiasma. Analysis of the relatively very small uncrossed pathway presents a problem which these experiments have not entirely solved. Eleven weeks after the operation, i.e. after the disintegration and partial resorption of the crossed retinal fibres, the persisting fascicle of normal uncrossed fibres could be traced along the outer surface of the optic tract from the chiasma to the level of the lateral forebrain bundle (Pl. 3, fig. 12). Here it became dispersed throughout the tract, and some of the fibres were seen to give collateral branches into the pars ventralis of the lateral geniculate nucleus in the rostral thalamus. Beyond this point the uncrossed fibres were indistinguishable from the persisting non-retinal components of the optic tract.

In material prepared between 12 and 26 days after the operation degeneration of the uncrossed fascicle could be followed in the homolateral optic tract, but again only to the level of the lateral forebrain bundle. Beyond this occasional fragments of axon debris were present in the otherwise normal tract, but the fibres could not be traced with precision. A careful search was made for terminal changes in the homolateral visual centres. In three specimens, those killed 10, 17 and 26 days after the operation, a few rings, bulbs and degenerating fibres were identified in the lateral neuropil of the pars ventralis of the lateral geniculate nucleus, but as these were not found in the remaining experimental material they must be regarded as of doubtful significance. No evidence could be found for the terminal distribution of uncrossed fibres in any of the other visual centres.

Marchi results

In the two specimens treated by the Marchi technique a quite considerable reaction was observed in the cerebral stump of the divided optic nerve. Myelin degeneration products were present in the form of droplets of various sizes and more rarely as ellipsoids. They were sufficient to give a general idea of the principal optic pathways.

The decussation of normal and degenerate bundles at the chiasma was clearly

shown, and a distinct Marchi reaction was present throughout the contralateral optic tract. Particularly large and numerous droplets occurred along the contralateral basal optic root, and could be followed as far as the nucleus opticus tegmenti within which there was scattered a profusion of minute osmiophilic particles. Myelin degeneration in the optic tract itself could be followed around the thalamus and into the stratum opticum of the tectum. Rows of droplets throughout the lateral geniculate and pretectal regions indicated the presence of retinal fibres, but this was, of course, insufficient to show whether any of the fibres ended there. A very slight reaction along the outer surface of the homolateral optic tract close to the chiasma was the only indication of the uncrossed fibres.

The results obtained by the Marchi method, although positive, clearly give no more than a general confirmation of the more detailed findings provided by use of the silver technique.

DISCUSSION

Former attempts to utilize the Marchi technique in reptiles met with varied success. Gross (1903), in a study of the reptilian chiasma, failed consistently to obtain a positive Marchi reaction after removal of an eye. Shanklin (1933) observed droplets of degenerating myelin in the contralateral basal optic root of the chameleon after removal of an eye, but was not concerned with the remaining visual pathways. Goldby (1937) obtained a moderate reaction in *Lacerta viridis* following certain forebrain lesions. The results achieved in the present work by means of the Marchi method compare favourably with those of earlier writers, and are as satisfactory as expected in view of the limitations of the method.

From the present observations on the visual pathways it is apparent that axonal degeneration in the lizard is for the most part very similar to that which occurs in mammals. The succession of changes corresponds to the sequence described by Ramón y Cajal (1928) as secondary or Wallerian degeneration. Such differences as do exist appear to be due to the relatively slow rate of degeneration in the reptile. Even at the high temperature used (30° C.) no changes can be detected less than 7 days after the operation and this is followed by a further interval of about 6 days during which the only change is a progressive increase in the affinity of the axons for silver. The subsequent stages of varicosity, fragmentation and resorption are the same as in mammals, except that they are slower in onset and more prolonged.

Failure to demonstrate terminal boutons in the visual centres of the normal lizard brain may have been due to several factors. It is possible that retinal fibres normally terminate in so-called free endings, or else in boutons so minute as not to be revealed by the technique adopted. Moreover, if boutons are normally present, it may be that they become readily stainable with silver only when degenerating, and this possibility is supported by the fact that the rings observed in the present experiments showed a progressive increase in their affinity for silver from the time of their first appearance. It is of interest to note that difficulty has sometimes been experienced in demonstrating normal boutons in the visual centres of mammals (Glees & Le Gros Clark, 1941; Nauta & van Straaten, 1947).

The question naturally arises whether the rings and bulbs observed in the lizard were in fact terminal structures, or whether they arose on the course of the terminal

arborizations of the retinal fibres. Their appearance in the available material suggests strongly that those seen from the 6th to the 10th day after the operation were predominantly and perhaps entirely terminal structures, but that from the 12th day they probably included many of the swellings and vacuoles which were seen to develop along terminal fibres. There is no doubt that fragmentation of such fibres would lead to the formation of rings and bulbs indistinguishable from those which were genuine nerve endings.

The organization of the reptilian visual system as disclosed by the experimental method is in some respects different from that revealed by the use of normal material.

In a recent paper (Ströer, 1939) it was submitted that in *Lacerta* each optic nerve consistently divides at the chiasma into two bundles, and that each bundle from the left nerve passes dorsal to the corresponding bundle from the right. A similar uniformity was indicated by Gross (1903) in a comparative survey of the reptilian chiasma. In the present investigation it was observed that the number of bundles into which the optic nerve divides is very variable, and also that a constant relationship does not exist between the bundles from the left and right nerves.

More important is the demonstration that some fibres of retinal origin do not cross at the chiasma. Such fibres are generally accepted as a mammalian characteristic, quantitatively related to the extent of the binocular field, and forming part of the anatomical basis of stereoscopic vision. Uncrossed fibres have been suspected in certain birds, but their existence has not been confirmed by experiments utilizing the Marchi technique (Harris, 1904). Partial decussation has never been described in reptiles, but the present experiments show that an uncrossed component is present in the optic tract not only of *Lacerta*, but of *Natrix* also. It remains for future work to demonstrate with precision both the origin and termination of these fibres. In the absence of this data their functional significance must be a matter of pure conjecture. It is tempting to relate them to the binocular field of vision which Kahmann (1935) has shown to exist in *Lacerta viridis* and in *Natrix*, but the problem clearly requires further investigation.

Systematic accounts of the visual system in reptiles all include reference to the basal optic root, which appears to be well developed throughout the class. That it contains crossed fibres of retinal origin was proved experimentally by Shanklin (1933) in the chameleon, and the present results show that in *Lacerta* it is composed entirely of such fibres. Widespread efferent connexions of the nucleus opticus tegmenti were described by Shanklin, including fibres to the oculomotor nucleus and medial longitudinal bundle. This, and the remarkable thickness of the fibres in the basal optic root, suggest that the tract functions, at least in part, as a rapid reflex pathway by means of which the extrinsic eye muscles are influenced by impulses of retinal origin.

There is repeated reference in the literature to a system of optic fibre bundles which are said to separate from the optic tract and pursue a course dorsally through the substance of the thalamus and pretectal region, subsequently rejoining the optic tract. The number of such bundles has been shown to vary considerably between species. Bellonci (1888) showed them as a conspicuous feature in the thalamus of *Natrix*, but they appear to be less obvious in *Varanus* (de Lange, 1913), *Alligator* (Huber & Crosby, 1926) and *Sphenodon* (Cairney, 1926). Later Huber & Crosby

(1933) described them in *Anolis* under the name of 'axillary optic tract', tracing some as far as the tectum before they rejoined the main optic tract. The present experiments indicate the relatively small extent of this system in *Lacerta vivipara*, and this may of course be simply a feature of the species. However, it is also clear that an extremely intimate relationship exists within the thalamus between these retinal bundles and the very numerous bundles from the pars dorsalis of the ventral supraoptic decussation, many of which also join the optic tract; indeed, the two are indistinguishable in normal preparations. This suggests the possibility that in studies based only upon normal material confusion between retinal and non-retinal fibres may have led to some exaggeration of the 'axillary' system.

This difficulty of distinguishing retinal from other fibres in normal material is well exemplified in a recent paper by Warner (1947) on the rattlesnake. In Warner's figure 8 it appears that the fibres labelled 'optic chiasma' actually belong to the ventral supraoptic (post-optic) decussation, while the massive basal optic root illustrated in figure 9 (Warner) is almost certainly composed mainly of the same fibres. This confusion of ventral supraoptic fibres with the basal optic root seems to have led to a misinterpretation of the structure labelled as the nucleus of the basal optic root in Warner's figure 11. The basal optic root itself is still present at a more caudal level (Warner's figure 12), where it is much smaller and obviously comparable to the tract so named not only in *Lacerta*, but also in other reptilian brains.

There has not been complete agreement in the past concerning the status of the reptilian lateral geniculate nucleus as a primary visual centre. Bellonci (1888), in *Natrix*, *Lacerta* and *Emys*, was unable to convince himself that fibres of retinal origin actually terminate in any part of the thalamus. Edinger (1899), using Golgi preparations, described fibres undergoing terminal ramification in the lateral geniculate nucleus of *Varanus*, and considered them to be of retinal origin. Similar observations were made by Beccari (1923) in *Lacerta muralis*. Huber & Crosby (1926), in *Alligator*, and Cairney (1926), in *Sphenodon*, confirmed that many fibres enter the geniculate nucleus from the optic tract, but the latter could follow stem fibres only into the dorsal part of the nucleus in the rostral thalamus. Later, however, Huber & Crosby (1933) expressed the view that the reptilian lateral geniculate nucleus 'serves more particularly as a place of synapse in the course of descending impulses from the tectum to lower centres', passing first along tecto-geniculate fibres and then through the fasciculus geniculatus descendens.

As a result of the present experiments it is established that a considerable number of fibres of retinal origin make synaptic contacts within the contralateral geniculate nucleus, all parts of it receiving both stem fibres and collaterals. Further, it may be that the pars ventralis, and perhaps the whole nucleus, also receives a small number of uncrossed fibres. There can be little doubt that the primary function of the nucleus is that of a relay centre for impulses of retinal and not of tectal origin. The disposition of the fusiform cell bodies and their dendrites, essentially in relation to incoming retinal fibres, necessitates this interpretation.

Retinal connexions with the pretectal region were suggested by Cairney (1926) who, in *Sphenodon*, followed collaterals from the optic tract into the nucleus geniculatus pretectalis. Huber & Crosby (1933), in *Anolis*, indicated connexions with the nucleus lentiformis mesencephali, and traced a fascicle from the optic tract into the

nucleus posterodorsalis. The experiments on *Lacerta* confirm that a large number of retinal fibres enter the pretectal region. Their terminal distribution is seen to be localized within the nucleus lentiformis and the nucleus geniculatus pretectalis, with the added possibility that a very small number reach the nucleus pretectalis. Although no connexion with the nucleus posterodorsalis was found, it may be significant that this nucleus appears to be more highly developed in certain other reptiles. The existence of pretectal visual centres is of particular interest after the demonstration that the pupil-light reflex in mammals is mediated by retinal fibres which terminate in that region (Hare, Magoun & Ranson, 1935; Magoun, Atlas, Hare & Ranson, 1936). Further experiment is necessary, however, before a specific function may be attributed to any part of this region in the reptilian brain.

Little is known about the precise terminal distribution of retinal fibres in the reptilian tectum. Extensive non-retinal connexions render specific analysis of the retinal components extremely difficult in normal material. Huber & Crosby (1926, 1933) described fibres turning from the stratum opticum to undergo terminal arborization in the stratum fibrosum et griseum superficiale. Cairney (1926), in *Sphenodon*, followed optic tract fibres not only into the stratum opticum, but also directly into the stratum fibrosum et griseum superficiale and into a surface layer corresponding to the stratum zonale. The present observations on *Lacerta* show that a large number of retinal fibres pass directly into the stratum zonale, and the extensive terminal reaction which occurred in this layer shows it to be an important area for the terminal distribution of such fibres. Within it they come into synaptic association not only with the small neurones which are found there, but also probably with long ascending dendrites from cells lying in deeper strata, including the periventricular layers. Such processes were described by de Lange (1913) and by Huber & Crosby (1933) in Golgi material, and probably account for many of the radially orientated fibres which were seen in the Nonidez preparations. The presence of retinal terminals superficial to the stratum opticum has also been observed in various mammals (e.g. Barris, Ingram & Ranson 1935; Jefferson, 1940; Nauta & van Straaten, 1947).

The sparsely scattered rings found in the stratum opticum suggest that at least a few retinal fibres end there, but the great majority undoubtedly enter the stratum fibrosum et griseum superficiale, where they terminate predominantly in the three outer substrata. It was therefore surprising to find relatively little positive evidence of terminal degeneration in this region, as compared with the striking changes in the lateral geniculate nucleus and pretectal region. Since the terminal ramifications of retinal fibres in this region are extremely fine, it is possible that they disappear without showing the structural changes which are typical elsewhere. Whatever the explanation, the fact remains that the principal evidence of retinal connexions in these layers of the tectum was negative, namely the widespread disappearance of neuropil.

In the light of the present results some comparison may be made between the organization of the reptilian visual system and those of Amphibia and mammals. Adequate experimental data for Amphibia are lacking at present, but the studies of Herrick (1948) on the normal amphibian brain indicate that the reception and correlation of impulses from the retina are primarily functions of the tectum. Optic terminals of less importance are described in the neuropil of the dorsal thalamus,

including the nucleus of Bellonci, showing a concentration in an ill-defined 'geniculate neuropil'. Other connexions are described with the hypothalamus and the pretectal neuropil, and fibres have been followed from the chiasma to the tegmentum in a somewhat diffuse basal optic tract.

In mammals, on the other hand, it is well known that the thalamo-cortical system has attained a dominant role in visual function at the expense of the midbrain. In the reptile, as typified by *Lacerta*, numerous retinal fibres certainly terminate within a well-developed lateral geniculate nucleus, and there are clearly-defined visual centres in the pretectal region; but the vast majority of the fibres reach the tectum which presents an extremely complex pattern of laminar differentiation. In addition, the basal optic root is well developed and terminates in a prominent tegmental nucleus. In general, the reptilian visual apparatus seems to be no more than an elaboration of the more primitive condition found in Amphibia, and to show no fundamental change in organization such as the 'prosencephalization' characteristic of mammals.

On the basis of comparable relations and assumed connexions it was proposed by Cairney (1926), and again more recently by Addens (1938), that the amphibian nucleus of Bellonci is represented in reptiles by the nucleus ovalis. In the present investigation no evidence was found to suggest that any retinal fibres end in the nucleus ovalis in *Lacerta*. Assuming the homology to be correct, it may therefore be supposed either that this nucleus has been abandoned as a thalamic visual centre in reptiles, or else that its retinal connexions (if they exist at all) arose as an amphibian specialization.

It is obvious that in a general way the diencephalic region of the reptilian brain which receives direct retinal connexions is comparable with the same region of the mammalian brain, where it forms the lateral geniculate body. Difficulties arise if more detailed comparisons are attempted.

Topographically the reptilian lateral geniculate nucleus is most closely comparable with the so-called ventral nucleus of the lateral geniculate body of mammals. This was indicated by Beccari (1923) and Papez (1935), who implied that the dorsal or principal nucleus of mammals is not represented in reptiles. The main efferent connexions of the lateral geniculate nucleus in reptiles appear to be through the fasciculus geniculatus descendens with the tegmentum of the midbrain, while geniculotectal fibres are also said to exist (e.g. by Huber & Crosby, 1933). Similar pathways have been described in relation to the ventral nucleus of the mammalian geniculate body (Rioch, 1929; Barris *et al.* 1935), but a fact which cannot be overlooked is that experimental studies on mammalian visual pathways have so far failed to provide unequivocal evidence for the ending of retinal fibres in that nucleus (Le Gros Clark, 1942).

Another possibility is that the reptilian geniculate nucleus might represent the dorsal nucleus of the mammalian geniculate body. Both are situated in the lateral part of the thalamus, and both receive direct retinal connexions. But it is well known that the dorsal nucleus in mammals functions essentially as a relay centre, projecting impulses of retinal origin through the optic radiations to the visual cortex. Geniculo-tectal fibres have also been described, but their existence is made doubtful by the experiments of Lashley (1934) and of Barris *et al.* (1935). In reptiles

there is no evidence of a forward projection from any part of the geniculate nucleus; on the contrary, its efferent fibres are directed caudally into the midbrain.

In some reptiles the lateral geniculate nucleus shows signs of differentiation into dorsal and ventral parts. This was observed in *Sphenodon* by Cairney (1926), who suggested that the dorsal and ventral parts might represent the dorsal and ventral nuclei of mammals. This view was taken by Le Gros Clark (1932), and by Huber & Crosby (1933). Dorsoventral subdivision of the geniculate nucleus is, however, a very variable feature in reptiles, and is ill-defined in most species. In *Lacerta*, for example, it is distinct only in the extreme rostral part of the nucleus, where the pars dorsalis might be considered as a rudiment of the dorsal nucleus of mammals. In favour of this interpretation is the observation that, unlike the rest of the nucleus, this part does not appear to contribute fibres to the fasciculus geniculatus descendens. Nevertheless, no forward projection has been demonstrated.

It is clear that the absence of evidence for telencephalic connexions from any part of the geniculate nucleus in reptiles makes it difficult to institute precise comparison with the dorsal nucleus of the mammalian geniculate body. Similarly, the absence of retinal connexions with the ventral nucleus of mammals is a difficulty in comparing this structure with the reptilian lateral geniculate nucleus, all parts of which receive abundant retinal fibres. One is forced to the conclusion that the visual system of reptiles is organized in a fundamentally different way from that of mammals, and is in fact more closely comparable with that of Amphibia. The general similarities between mammals and reptiles, such as the presence in both of thalamic, pretectal and tectal retinal connexions, would be perfectly consistent with an independent derivation of both from generalized amphibian ancestors.

SUMMARY

1. A description has been given of the normal histological appearance of thalamic, pretectal and midbrain centres related to the optic tracts in *Lacerta vivipara*. They are compared with corresponding structures described in other reptiles.

2. Silver impregnation of the brain after removal of an eye has revealed axonal and terminal degenerative changes, essentially similar to those which occur in mammals, except that they develop more slowly.

3. Twenty brains prepared by silver impregnation and two treated by the Marchi method have been utilized for the study of the course and distribution of degenerated fibres of retinal origin. Evidence was obtained for the following main conclusions:

(*a*) That the optic nerve contains only afferent fibres.

(*b*) That decussation of the optic nerves is almost, but not quite, complete.

(*c*) That crossed retinal fibres terminate in the lateral geniculate nucleus, in the nucleus geniculatus pretectalis, in the nucleus lentiformis mesencephali, in the superficial layers of the optic tectum (including the stratum zonale) and in the nucleus opticus tegmenti. A few may also end in the nucleus pretectalis.

(*d*) That the basal optic root is composed entirely of crossed retinal fibres.

(*e*) That uncrossed retinal fibres form a small fascicle on the outer surface of the optic tract, some probably having connexions in the rostral thalamus with the pars ventralis of the lateral geniculate nucleus.

(*f*) That as the optic tract runs on the lateral surface of the thalamus it contains in its deeper part numerous non-retinal fibres. Many of these appear to cross in the ventral supraoptic decussation.

4. The organization of the visual system in *Lacerta* has been compared with those in Amphibia and mammals. The possibility of homology has been discussed, with special reference to the lateral geniculate nucleus.

This work has been carried out under the direction of Prof. F. Goldby of the Department of Anatomy, St Mary's Hospital Medical School. I should like to express my thanks for the advice and criticism which he has given throughout.

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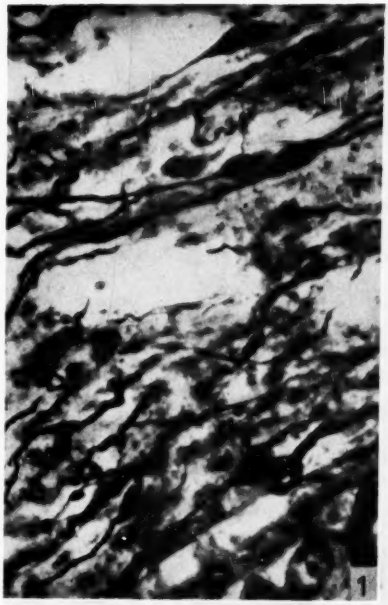
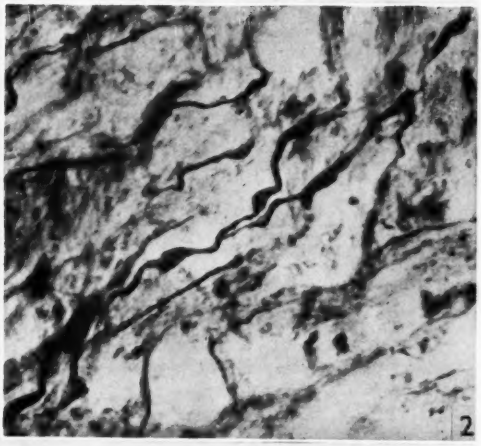
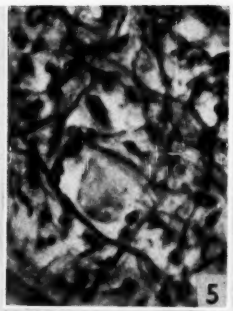
ABBREVIATIONS

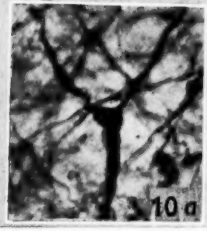
Bas.opt.rt.	Basal optic root		
Col.inf.	Colliculus inferior		
Dec.supraopt.dors.	Decussatio supraoptica dorsalis		
Dec.supraopt.vent.p.dors.	Decussatio supraoptica ventralis pars dorsalis		
Dec.supraopt.vent.p.vent.	Decussatio supraoptica ventralis pars ventralis		
Fas.gen.desc.	Fasciculus geniculatus descendens		
Fas.gen.pret.desc.	Fasciculus geniculatus pretectalis descendens		
L.F.B.	Lateral forebrain bundle		
n. III	Nervus occulomotorius		
n. IV	Nervus trochlearis		
N.dors.lat.ant.	Nucleus dorsolateralis anterior		
N.dors.med.ant.	Nucleus dorsomedialis anterior		
N.gen.lat.	Nucleus geniculatus lateralis		
N.gen.lat.(lat.)	Nucleus geniculatus lateralis (lateral neuropil)		
N.gen.lat.(med.)	Nucleus geniculatus lateralis (medial cell plate)		
N.gen.lat.p.dors.(med.)	Nucleus geniculatus lateralis pars dorsalis (medial cell plate)		
N.gen.lat.p.vent.(med.)	Nucleus geniculatus lateralis pars ventralis (medial cell plate)		
N.gen.pret.	Nucleus geniculatus pretectalis		
N.gen.pret.(lat.)	Nucleus geniculatus pretectalis (lateral neuropil)		
N.gen.pret.(med.)	Nucleus geniculatus pretectalis (medial cell plate)		
N.lent.mes.	Nucleus lentiformis mesencephali		
N.opt.teg.	Nucleus opticus tegmenti		
N.paravent.	Nucleus paraventricularis		
N.perivent.hyp.	Nucleus periventricularis hypothalami		
N.post.	Nucleus posterodorsalis		
N.pret.	Nucleus pretectalis		
N.rot.	Nucleus rotundus		
N.supraped.	Nucleus suprapeduncularis		
N.vent.	Nucleus ventralis thalami		
Tect.opt.	Tectum opticum	*Tr.opt.(deg.)*	Tractus opticus (degenerate)
Tr.opt.	Tractus opticus	*Vent.lat.*	Ventriculus lateralis

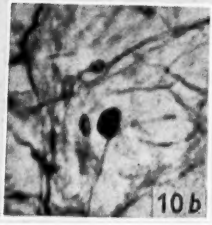




ARMSTRONG—Study of the visual pathways in a reptile (*Lacerta vivipara*)







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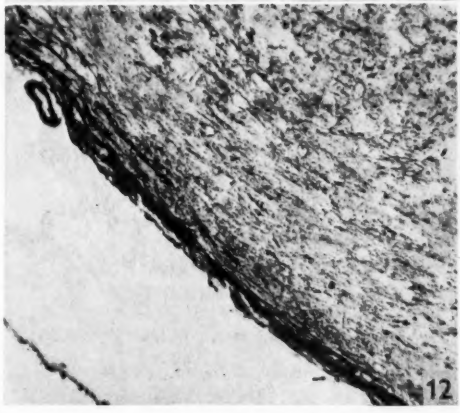

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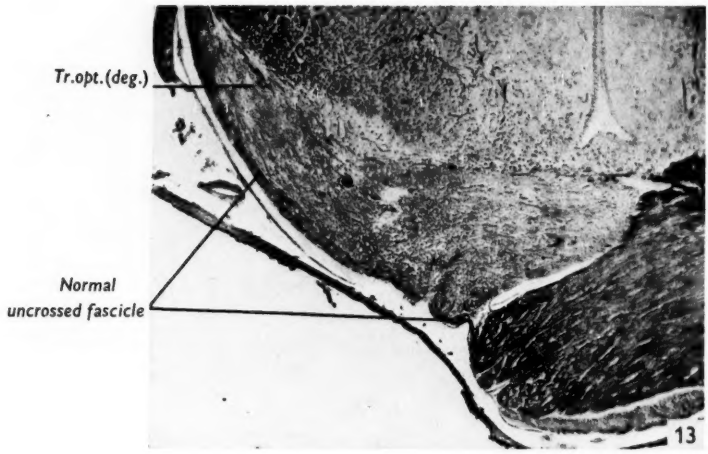

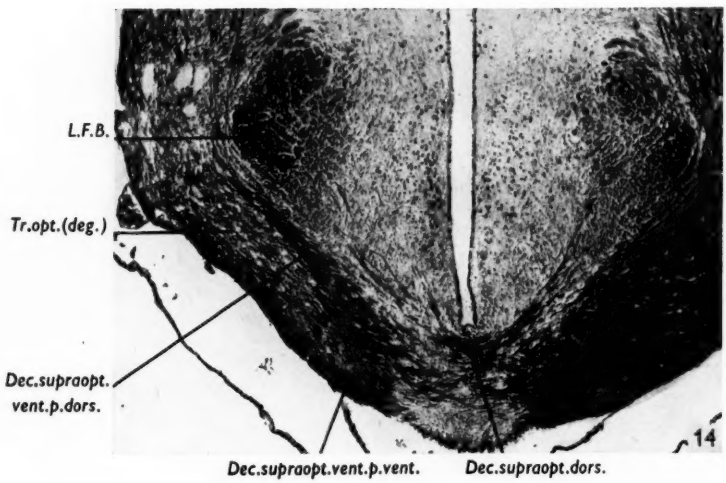

ARMSTRONG—Study of the visual pathways in a reptile (*Lacerta vivipara*)

EXPLANATION OF PLATES

Plate 1

Fig. 1. Axonal degeneration in the right optic tract 15 days after removal of the left eye. The fibres are tortuous, and present irregular and fusiform swellings. Degeneration vacuities are to be seen in the upper part of the field. Nonidez' method. (×1340.)

Fig. 2. Axonal degeneration in the right optic tract 25 days after removal of the left eye. Short lengths of distorted axon are seen to be fragmenting. Nonidez' method. (×1340.)

Fig. 3. Sagittal sections of (*a*) the left basal optic root, and (*b*) the right basal optic root, 15 days after removal of the left eye. The homolateral tract is normal; the contralateral tract shows advanced degeneration. Nonidez' method. (×750.)

Plate 2

Fig. 4. Terminal rings in the lateral neuropil of the lateral geniculate nucleus 7 days after removal of the opposite eye. One ring shows thickening and a single bulb is seen in the lower part of the field. Nonidez' method. (×1340.)

Fig. 5. Rings and bulbs in the lateral neuropil of the lateral geniculate nucleus 12 days after removal of the opposite eye. Some rings are seen in relation to a cell body (cytoplasm unstained). Nonidez' method. (×1340.)

Fig. 6. Terminal degeneration in the lateral neuropil of the lateral geniculate nucleus (pars ventralis in the rostral thalamus) 13 days after removal of the opposite eye. Note the profusion of rings and bulbs, and the increased proportion of the latter. Nonidez' method. (×1340.)

Fig. 7. Terminal degeneration in the nucleus lentiformis mesencephali 13 days after removal of the opposite eye. Rings and bulbs are applied to a process of a large fusiform cell. Nonidez' method. (×1340.)

Fig. 8. Large terminal bulbs in the nucleus opticus tegmenti 13 days after removal of the opposite eye. Nonidez' method. (×1340.)

Fig. 9. Terminal rings and bulbs in the stratum fibrosum et griseum superficiale of the optic tectum 13 days after removal of the opposite eye. Nonidez' method. (×1340.)

Fig. 10. The stratum zonale of the optic tectum 20 days after removal of the opposite eye. (*a*) Terminal optic fibre swollen at the point of division. (*b*) Large terminal bulb. Nonidez' method. (×1340.)

Plate 3

Fig. 11. Degenerating fascicle of uncrossed retinal fibres turning on to the outer surface of the homolateral (normal) optic tract. Left eye removed 13 days previously. Nonidez' method. (×940.)

Fig. 12. Persisting fascicle of normal uncrossed fibres on the outer surface of the degenerate optic tract 11 weeks after removal of the opposite eye. Nonidez' method. (×200.)

Plate 4

Fig. 13. Transverse section of optic chiasma of the grass snake (*Natrix*) 19 weeks after removal of an eye. Uncrossed retinal fibres are seen as a normal fascicle turning on to the outer surface of the degenerate optic tract. Nonidez' method. (×85.)

Fig. 14. Transverse section (*Lacerta*) just caudal to the chiasma 11 weeks after removal of the left eye, showing the disposition of the supraoptic decussations. Nonidez' method. (×80.)

A CRITICAL EVALUATION OF METHODS USED TO DEMONSTRATE TISSUE NEURAL ELEMENTS, ILLUSTRATED BY REFERENCE TO THE CORNEA

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INTRODUCTION

A review of the literature on the anatomy of the finer details of nerve fibres and endings suggests that not sufficient regard has been paid to the possibility that the technical procedures used in the preparation of specimens for microscopical examination may themselves have been responsible for some of the variations in the pictures described.

In view of this we have recently taken the opportunity of re-investigating this problem in the course of a study of the innervation of the cornea. The cornea is particularly favourable for a study of this kind, since its transparency permits an examination of its neural elements in fresh preparations, and even in the living eye.

In the course of our investigation we have employed all those techniques described in the literature available to us which have been used for demonstrating corneal neural elements, together with a variety of modifications which suggested themselves to us in the course of our work.

In the paper which follows the pictures obtained by these various histological procedures are compared and contrasted with one another and with the pictures of normal living nerve fibres seen through the slit lamp microscope. In conclusion, an attempt has been made to evaluate the methods used in relation to their employment for staining tissue neural elements in general.

REVIEW OF THE LITERATURE

It is not proposed to give a comprehensive review of the literature in this section, but rather to select papers indicating the gradual development of technical methods that now enable nerve fibres in the cornea to be demonstrated specifically and selectively. Certain important technical publications are more appropriately discussed in relation to our own observations and will be found in the section headed 'Techniques employed'.

In 1831 Schlemm, by ordinary dissecting methods, demonstrated that nerve fibres reach as far as the corneo-scleral junction, but the presence of nerve fibres in the cornea itself was disputed until the advent of histological methods.

The conclusive demonstration that nerve fibres ramify throughout the cornea proper followed the use of dilute acetic acid as a histological reagent by Purkinje (1845), Bochdalek (1849), Luschka (1850), Ciaccio (1863) and others. It is worth quoting a statement made by Ciaccio at this early date, for it shows that the very problems in neuro-histology which are still a source of controversy to-day were even

* Holding a grant from the Swiss 'Stiftung für biologisch-medizinische Stipendien'; on leave from the Neurosurgical Clinic, University of Zürich.

then clearly recognized: '. . . difficulty arises from the peculiar structure of the cornea itself which contains a very large number of corpuscles with many anastomosing processes. This undoubtedly causes much difficulty in tracing the ultimate nerve-fibres running through its laminated structure and if we do not use much diligence in observing we may mistake the processes of the cornea-corpuscles for the finest nerve-fibres and draw the erroneous conclusion that there exists an intimate connexion between the nerve-fibres and the cornea-corpuscles.'

In 1867 Cohnheim, in a classical paper, introduced the gold-chloride impregnation method and opened a new era in neuro-histology. He demonstrated conclusively for the first time the presence of nerve fibres in the corneal epithelium. Following this, Klein (1871, 1880), Hoyer (1873) and Ranvier (1881), also using gold chloride, extended and to some extent disputed Cohnheim's observations. In these papers details are given which led to improved methods of impregnation. Following the introduction of other staining methods the gold-chloride technique gradually lost its popularity, but was adopted by Nageotte (1911*a–c*), Nageotte & Guyon (1926) and Boeke (1925, 1935) for staining corneal nerves.

Methylene blue was introduced by Arnstein (1887) and Dogiel (1890) to stain the nerve fibres of the cornea. The excellence of Dogiel's preparations is apparent from his illustrations, and it is true to say that his observations form the basis upon which all later work has been built. Following Dogiel a number of workers gave technical details of the different ways in which they employed methylene blue for staining corneal nerves, and among them may be mentioned Hosch (1891), Agababow (1912), Attias (1912) and Nakajima (1930). In fact, the classical paper of Attias, upon which is founded the present-day conception of the innervation of the cornea, was based mainly on the observation of preparations stained with methylene blue.

Silver methods have tended to supersede methylene-blue staining in modern times. Cajal (1909) and Bielschowsky & Pollack (1904) both described the innervation of the cornea, but made no specific contributions which were in any way at variance with what had gone before. It seems that the main reason for adopting silver methods, in preference to methylene blue, was because interest was shifting away from the topographical distribution of nerve fibres towards the problem of the possible connexion between nerve fibres and their terminals and the cells of the tissues in which they ramify, a cytological problem which demands better tissue fixation than can ordinarily be obtained in preparations stained with methylene blue.

Three other names should be mentioned in connexion with silver staining of the cornea: Boeke (1925), Reiser (1935–6) and Martinez (1940). Reiser used preparations of the cornea stained by silver methods as arguments against the validity of the neuron theory.

Other technical methods have been used in the study of corneal innervation; for instance, Bach (1896) and Fritz (1904) used a silver, osmic acid, bichromate mixture (after Cajal) in studying the pattern of corneal innervation, and Ernyei (1934) used both osmic acid and silver methods in the hope of demonstrating myelinated nerve fibres and ganglion cells. However, no new facts were contributed by these methods. Nageotte and Nageotte & Guyon used special methods of their own devising to demonstrate the nuclei and cytoplasm of the Schwann cells, which did not at the same time stain the nerve axis cylinder.

From 1912 onwards, a new approach to the study of the innervation of the cornea was adopted as the result of the introduction of the slit-lamp and corneal microscopes into ophthalmological practice. The first paper on the subject was by Wolff (1912), who described the appearance of corneal nerves, using the slit-lamp microscope. Erggelet (1914) confirmed Wolff's observations, but added nothing new. In 1919, Koeppe gave a fuller description of the slit-lamp appearances of corneal nerves and, in fact, claimed that his observations were in accordance with the descriptions of Attias, who used excised preparations stained with methylene blue.

In 1922, Knüsel & Vonwiller combined methylene-blue staining and 'slit-lamp' technique in examining normal and pathological corneas. They again confirmed the findings of Attias. Their staining technique with methylene blue was carried out by the simple procedure of instillation of a few drops of 1% of the dye into the conjunctival sac. This technique was also used and, to some extent, developed by Nakajima.

So far, however, no new facts concerning the anatomy of corneal innervation have resulted from the use of the slit-lamp or corneal microscopes. This is probably due partly to the low magnifications used and partly to Attias's work having been considered exhaustive. However, the slit-lamp appearances after methylene-blue staining have proved of value in studying corneal pathology, the effects of operative interference on the corneal nerves, and corneal nerve degeneration and regeneration. Jent (1945), for instance, studied corneal nerve regeneration by this method.

So far we have been unable to find any publications reporting the use of phase-contrast microscopy in the study of the neural anatomy of the cornea.

It is clear from the papers reviewed that each observer considers that the technique employed is adequate for the purpose for which it was used, and, moreover, it appears to be assumed that in general the observations made represent the state of affairs in the living cornea. No individual authors have compared the pictures obtained by a variety of histological methods. In particular, there has been no critical comparison of pictures of living nerve fibres seen through the slit-lamp and phase-contrast microscopes with those obtained from tissues prepared for microscopical examination.

TECHNIQUES EMPLOYED

I. Methylene blue

We have employed most of the techniques which have been described, and have finally chosen methylene blue as our chief weapon, for in our hands it has given the best all-round results. We shall, therefore, describe our experiences with methylene blue first.

The technique now employed by us has been the result of numerous trials, the object of which has been to evolve a method that is absolutely reliable and that gives a picture corresponding as closely as possible to that seen in the living cornea under the slit-lamp microscope before, and under the corneal microscope after, methylene-blue staining.

In order to give a clear idea of the difficulties involved in obtaining perfect specimens it is, we think, necessary to describe the results obtained by a variety of

methods which for one reason or another did not prove entirely successful, and also to discuss our observations in relation to those obtained by other workers. This is, we feel, the more important since, although methylene blue can in one sense be described as a universal nerve stain, to achieve reliable results and complete staining without distortion every tissue has to be treated in a slightly different manner. To give but one example, when staining the cornea of the dog-fish (*Scyllium canicula*) it is necessary to make up the methylene blue in a sodium chloride solution which is isotonic with the anterior chamber fluid of this sea-water vertebrate.

(1) *Methods of introducing the dye into the tissue*

(*a*) *Intravenous drip*

The success of the intravenous drip technique for staining the peripheral nervous system (Feindel, Sinclair and Weddell, 1947) suggested that this would also be the method of choice for the cornea. Nevertheless, compared with the supravital methods employed long ago by Dogiel, Agababow and Attias, it gave disappointing results. The method stained well the nerves in the sclera and at the corneo-scleral junction and nerve fibres passing for about 1 mm. from the limbus towards the centre of the cornea, but few nerve fibres could be traced to the centre of the cornea itself and these were poorly differentiated. The nerve fibres stained were those in the region of blood vessels, the nerve fibres in the avascular cornea itself being hardly stained at all. This result was somewhat surprising in view of the observations reported by Arnstein, by Agababow and by Hosch, the last of whom, at the suggestion of Ehrlich (1886), injected between 1 and 4% methylene-blue solution through the heart and claimed to have obtained even staining throughout the cornea.

When intravenous methylene blue is employed, the lacrimal secretion is blue in colour towards the end of the perfusion, and the corneal nerves in those eyes which happen to remain closed during the operation are evenly, though faintly, stained throughout. This suggested that the local instillation of methylene blue into the conjunctival sac should next be tried, or possibly a combination of this with an intravenous injection.

(*b*) *Instillation of methylene blue into the conjunctival sac*

This method was used by Agababow in rabbits. He used solutions of methylene blue in water of between 0·2 and 1% and instilled a few drops periodically into the conjunctival sac. He then removed the cornea for microscopical examination after 'fixation' of the dye with ammonium picrate.

Knüsel (1923) instilled between 0·5 and 1% of the dye into the conjunctival sac of normal and pathological eyes in man and then examined the cornea under the slit-lamp microscope. He stated that the nerves were well stained after 15 min. and remained blue for several hours. He also made the interesting observation that if dyes were employed which before use had been kept for some time after receipt from the manufacturer, the patients complained of far less pain than when freshly manufactured dyes were used. This method was also employed by Nakajima, who used between 0·3 and 1% of methylene blue manufactured by Grübler. Nakajima, however, used to scarify the cornea in animals before staining it.

Jent used methylene blue for corneal nerve staining in rabbits, but introduced powdered dye into the conjunctival sac, which dissolved in the tears. By this means he hoped to obtain a solution resembling in isotonicity the lacrimal fluid which would assist in the penetration of the dye.

It was recognized by a number of authors that, although the instillation method stains the corneal nerves in the epithelium and the immediately subjacent tissue fully, the nerves in the deeper layers of the cornea are not well stained.

Fischer (1929), as the result of experiments on the properties of the living cornea in relation to the diffusion through it of a number of different dyes as well as a number of specific ions, came to the conclusion that dyes which are basic in nature diffuse through the undamaged cornea virtually from within outwards only; on the other hand, acid dyes pass mainly in the reverse direction. Methylene blue, being a weak basic dye, is, he calculated, likely to penetrate into the anterior chamber in a ratio of only 1 part in 600,000. It is probably for this reason that the instillation of methylene blue into the conjunctival sac does not stain nerves in the deeper layers of the cornea.

Holt & Cogan (1946) measured the electrical 'impedance' of the excised cornea and found that it was considerable as long as the epithelium was present. The 'impedance' of the stroma, however, was low. They concluded from their experiments that, in so far as 'impedance' is an index of ionic permeability, the epithelium would offer considerable resistance to the passage of ions, whereas the stroma would have a resistance little more than that offered by an equivalent volume of isotonic sodium chloride solution. Thus the corneal epithelium is probably an even more effective semi-permeable membrane than previous diffusion measurements have shown it to be.

Although it is possible to use the instillation method for certain purposes with excellent results, it must be remembered that one can never stain all the corneal nerves in this way. The reason that the nerves are stained at all can be accounted for only by the fact that the concentration of dye necessary to stain nerves is very low indeed. It is, of course, possible that the dye 'damages' the corneal epithelium very slightly, which thus enables it to penetrate for a short distance beneath the epithelium, for Knüsel has shown that the dye penetrates more rapidly when corneal damage is demonstrable.

We have employed the following methods to demonstrate the superficial nerve fibres in the cornea for examination under the slit-lamp and corneal microscopes. These methods do not differ from those employed by Knüsel, save that the dye used by us was manufactured by Merck.* In rabbits a solution of 1–2 % of dye in normal saline solution is instilled into the conjunctival sac, and in 15 min. many cornea nerves are well stained. At the end of 30 min. more axis cylinders have taken up the dye and staining is as complete as the method will allow. The rabbit's cornea does not appear to be damaged by these concentrations (as judged by slit-lamp appearances) and no nociceptive responses can be observed as the result of the instillation. We have also used powdered dye, placed in the conjunctival sac, and obtained equally good results. In order to study the nerve fibres and terminals so stained to best advantage through the corneal microscope we have used albino rabbits and

* The dye used by us throughout this investigation and referred to as methylene blue was manufactured by the Merck Institute, Rahway, New Jersey, U.S.A., and is called Methyl Thionin Chloride (U.S.P.).

instilled pilocarpine into the eyes before commencing the experiment. Pilocarpine has no demonstrable effect on the morphological appearance of the nerves or their terminals.

In man 0·25–0·5 % of methylene blue in normal saline solution, when instilled into the conjunctival sac, gave rise to no pain nor other sensory phenomena, and was found to stain the corneal nerves faintly. Good staining, however, follows the instillation of 1–2 % of the dye, but these concentrations arouse an unpleasant burning sensation which can be partially allayed by the instillation of 1 % procaine a quarter of an hour before the instillation of the dye. The conjunctiva remains 'injected' for 1–2 hr. after using the higher concentrations of dye. It is of some interest that the burning sensation does not become apparent for from 3 to 5 min. after instillation of the dye, even if no anaesthetic is used previously.

In order to obtain more information concerning the staining properties of methylene blue it was thought worth while to try the effects of immersing the excised cornea in dye solutions of various compositions.

(*c*) *Supravital staining*

The following three methods were employed:

The first was that suggested by Attias. This consists of immersing the excised cornea in 0·01 % methylene blue in normal saline for between 1 and 3 hr. in an incubator at 37° C. The cornea can be removed from time to time and examined under the microscope, and the progress of staining noted. Attias found that to get even distribution of the dye solution over the cornea certain precautions had to be taken, for there is a natural tendency for the cornea to float if merely immersed in the solution; to overcome this he placed the cornea between layers of glass-wool soaked in the dye solution.

The second method, suggested in the first place by Dogiel, is very similar save that the cornea is not immersed in the dye but is placed on a glass slide after excision, a few drops of dye being instilled into its concavity which is orientated uppermost. Agababow's modification has also been tried. His method is very similar to that of Dogiel, except that an anterior segment of the eye which retains both lens and iris is removed and suspended by threads in the form of a hemisphere, into which the dye is instilled. In both cases the preparations are placed in an incubator at 37° C. and the progress of staining determined by repeated examination under the microscope.

The third method that we tried was that advocated by Heller, Thomas & Davenport (1947). A fresh cornea is excised and placed in a 0·01 % solution of methylene blue in normal saline buffered with phosphate to a pH of 5·6. As before, the preparation is kept at 37° C., but in this instance oxygen is bubbled through the staining mixture. The cornea remains in this turbulent solution for between 2 and 4 hr., being removed every half hour and examined under the microscope, the progress of staining being noted.

Although these methods certainly stained the nerve fibres, the results were neither as specific nor as consistent as those obtained by intravital techniques. In particular, we found that general tissue staining was always pronounced when it was judged that

the nerve fibres were adequately stained, and that the nerve fibres always appeared to be considerably distorted when compared with similarly stained fibres in living animals when examined under the corneal microscope.

(*d*) *Injection of methylene blue into the anterior chamber of the eye*

This method has not been employed to any great extent as far as we are aware. Andogsky (1897) stated that nerve fibres in the iris can be stained by methylene blue when it is injected into the anterior chamber of the eye at a concentration of 1·5 %, but he made no mention whether corneal nerves were also stained by this procedure.

We have employed this method to great advantage in staining corneal nerves, and we believe that when the technique outlined below is carried out carefully, corneal nerve axis cylinders are stained more specifically and with less distortion than by any other method. They are also stained uniformly and the technique gives consistent results. We have used the method successfully in a variety of vertebrates, the dogfish (*Scyllium canicula*), the frog, the guinea-pig, rabbits of many breeds, the monkey (*Macaca mulatta*) and man.

The details of the procedure in the case of the rabbit are as follows:

The animal is anaesthetized with nembutal given intravenously. A 0·125 % solution of methylene blue in normal saline solution is taken up into a warm syringe (37° C.) fitted with a fine hypodermic needle. The needle is then inserted into the anterior chamber and 0·05 c.c. of aqueous humour is withdrawn and mixed with an equal quantity of the dye; 0·05 c.c. of the resulting mixture is then slowly injected into the anterior chamber. The concentration of the dye injected is thus approximately 0·06 %. 5 min. after injection, stained nerve trunks and finer ramifying nerve fibres can be seen clearly under the corneal microscope. In fact, it is possible to see the general pattern of distribution of the corneal nerves at this time and even to trace some of the terminal ramifications of the finest fibres throughout their length. The dye is allowed to remain in the anterior chamber for 30 min., after which the animal is killed and the cornea removed for 'fixation' and clearing. Although the nerve fibres are stained soon after the injection of the dye, it is difficult to 'fix' the dye in its blue form in the fibres for histological examination unless the staining process is allowed to proceed for about 30 min. Fading always occurs rapidly after removal of the cornea in specimens in which the dye is left in the anterior chamber for a short time only. If, on the other hand, staining is prolonged beyond 30 min., the nerve fibres are already colourless by the time the specimen has been removed for examination. On occasions air was accidentally injected along with the dye into the anterior chamber. If anything, this seems to improve the staining; it certainly has no adverse effect. To obtain maximum differentiation of the most superficial nerve fibres in the cornea a few drops of 1 % methylene blue were instilled into the conjunctival sac during the staining process.

Certain points in the technique need emphasis so that consistently good results may be obtained:

(i) The staining mixture must *not* be injected under pressure. If the pressure is even *slightly* raised, Descemet's membrane ruptures and the cornea becomes cloudy, for fluid infiltrates into the substantia propria and the whole cornea appears opaque

and dark blue in colour. Nerve fibres are certainly stained under these conditions, but are often distorted through stretching, rupture or shrinkage. Artefacts so produced may easily be mistaken for complicated end-organs. Corneal cells are also darkly stained and so confuse the picture obtained.

(ii) It cannot be too strongly emphasized that the cornea must be handled with the greatest care until it is finally mounted, to avoid distortion of nerve fibres and the production of bizarre artefacts. It is particularly important that the cornea be removed carefully after the staining process has been completed. We have found the following method to be satisfactory.—An incision is made into the sclera with a sharp razor and the cornea is then excised with fine sharp scissors by cutting around the corneo-scleral junction. It is even dangerous to remove the eyeball from the orbit before excision of the cornea, unless the walls of the orbit are removed and the globe detached without deformation.

(iii) If the concentration of dye used is much in excess of 0·06 % and, moreover, injected rapidly into the anterior chamber, dye deposits in the form of needle-like crystals cover the internal surface of the cornea and also appear in the substantia propria so that the nerve fibres are obscured. In addition, the cellular and fibrous elements of the substantia propria become stained, and it is difficult to distinguish nerve fibres from the stained cytoplasmic processes of Schwann cells and 'corneal corpuscles', and even from Schwann cell nuclei.

(2) *The preparation of the tissue for microscopic examination*

It is fortunate that nerve fibres and their terminals can be examined in the living cornea through the corneal microscope, for it is thus possible to determine the quantity of dye lost and the degree of distortion which takes place during the process of 'fixation', dehydration and clearing. Using this as a test method, we have found that very adverse effects, such as loss of dye and gross distortion, may take place unless the after-treatment is both suitable for the particular tissue under consideration and carefully carried out. We have employed a number of methods, many of which have proved unsatisfactory, but nevertheless it is proposed to describe these procedures in some detail in order that the principles involved may be made clear; for although we have now evolved a method of 'after-treatment' for the cornea which is satisfactory, it does not follow that this method can be used without modification when preparing other tissues in which it is desired to study the neural histology.

The first method we used was that finally adopted by Dogiel, and which has been employed by the majority of workers since then (see Weddell & Glees, 1941). The dye is 'fixed' by immersing the cornea in a saturated solution of ice-cold ammonium molybdate for 4–12 hr. after which it is washed for half an hour in running tap water. Dehydration is carried out quickly by immersion in ice-cold commercial absolute alcohol (*c.* 10 min.), followed by a further brief period (*c.* 10 min.) in ice-cold redistilled absolute alcohol. The tissue is cleared by immersing in pure benzol and is subsequently stored in pure methyl-salicylate or mounted in Canada balsam. Preparations tend to fade if stored in methyl-salicylate, but when mounted in balsam they often remain unchanged for many months or even for years. Despite the brief time

of immersion in alcohol much dye is lost during this stage, particularly during the first stage in commercial absolute alcohol.

Not every tissue will stand immersion in absolute alcohol straight from water without gross shrinkage, but if lower concentrations of alcohol are used before the absolute stage most of the dye is extracted.

The shrinkage resulting from immersion in absolute alcohol after washing distorts corneal nerve fibres; many rupture and many, seen to have a smooth contour under the corneal microscope, contain large irregular beads along their course after this treatment. If tertiary butyl alcohol is used in place of ethyl alcohol, much less dye is removed from the axis cylinders, but this advantage is offset by the even greater distortion due to extreme shrinkage. Although tertiary butyl alcohol is not the best agent to use for dehydrating the cornea following methylene-blue staining of nerve axis cylinders, it has proved of value in other tissues, for instance skin.

A modification of this method employs 'fixation' in Bethe's (1896) solution and is found to give somewhat better results in that less dye is extracted by the alcohol. It has the following disadvantage, however, that both the nerve fibres and the surrounding tissues are stained a brownish colour and thus the differentiation between them is less distinct.

The second method we used is slightly modified from that advocated by Attias. The dye in the tissue is 'fixed' by placing it in a saturated solution of ammonium picrate for 10–20 min. and then, without washing, for 5 hr. in a saturated solution of ice-cold ammonium molybdate. The tissue is then transferred to a mixture of a saturated aqueous solution of ammonium picrate and pure glycerine in equal proportions. In 10–12 hr. clearing is complete and the tissue can be transferred to pure glycerine for storing. This method has proved highly successful. Very little dye is lost, as judged by comparison with corneal microscopic appearances, and there is no distortion of the nerve fibres. The tissue, however, is never so transparent, and thus the nerves never so sharply differentiated from the surrounding tissues, as when alcohol dehydration is used. The tissue appears yellow in colour following this treatment and the nerve fibres appear violet. The specimens prepared in this manner can be usefully studied only for between 12 and 14 days, after which the dye leaves the nerve fibres and diffuses into the storage fluid. Diffusion of the dye from the nerve fibres is delayed for a period of between 3 and 4 weeks if Schabadasch's (1930*a*, *b*) modification is employed. The tissue, instead of being stored in glycerine, is immersed in a solution made by placing an excess of ammonium picrate crystals in chemically pure glycerine at 25° C. for 6 days. Even more permanent preparations can be made by mounting the tissue in a similar solution to which gelatine has been added. Recently, we have mounted specimens in Canada balsam, after they have been stored in the ammonium picrate and pure glycerine mixture for some days, and obtained preparations which we believe will remain virtually permanent.

The third method we tried was that recommended by Schabadasch. The tissue after staining is placed in a solution of 1–2% ammonium iodide to which has been added ammonium picrate up to saturation point. The tissue remains in this solution from 3 to 6 hr., after which it is transferred to a saturated aqueous solution of ammonium picrate and glycerine, in which it is stored. The nerve fibres are

stained violet after this treatment, but it is very difficult to distinguish the fine nerve fibres clearly from other stained tissue elements.

So far we have described methods of after-treatment used to obtain whole preparations of the cornea. It is possible to obtain sections after staining with methylene blue although it is technically difficult. Frozen sections are cut from the stained cornea after fixation of the dye in either ammonium molybdate or ammonium picrate. The sections are washed, dehydrated in ice-cold redistilled absolute alcohol, cleared in benzene and mounted in Canada balsam. The use of wax or other embedding methods is not very satisfactory for there is a greater loss of dye from the nerve fibres due to the necessary duplication of the dehydration process.

In summary, therefore, we can say that for critical studies of corneal innervation following methylene-blue staining it is necessary to treat the excised and stained cornea as follows. The tissue is placed in a saturated solution of ammonium picrate for 10–20 min. and then transferred without washing into a solution of ice-cold ammonium molybdate for about 5 hr. The tissue is then placed in a mixture of a saturated aqueous solution of ammonium picrate and pure glycerine in equal proportions for 10–12 hr. The tissue is finally immersed in a solution made by placing an excess of ammonium picrate crystals in pure glycerine at 25° C. for 6 days. After a week in this solution it can be mounted in Canada balsam for examination at leisure.

It is important that the *prepared* cornea be carefully mounted for microscopical examination for, if it is forcibly flattened, there is considerable danger that nerve fibres will be distorted or ruptured and even complicated 'nerve terminals' created. We usually make three equally spaced radial incisions (using sharp scissors) extending half the distance between the centre and periphery of the cornea. The tissue is then laid on a glass slide and a cover-slip placed over it. It will be found to flatten sufficiently for examination if the cover glass is gently pressed upon. If incisions are made into the cornea before it has been prepared as outlined above, artefacts are invariably seen.

(3) *Methods of improving the intensity of staining*

Mention must now be made of the work of Schabadasch (1930*a*, *b*, 1935), who used a variety of methods to improve the quality of nerve staining by methylene blue. His preparations were always made by the intra-arterial injection of the dye, and he believed that for successful staining by this method the following points were of considerable importance. The pH of the injected fluid must be on the acid side, preferably around 5·6, and can be obtained by using phosphate buffers. Heller, Thomas & Davenport also subscribed to this view, using an immersion technique. Schabadasch believed that better differentiation between stained nerve fibres and surrounding tissues was obtained by the use of hydrogen acceptors, such as resorcin or pyrocatechin, etc., and he always added small quantities of one of these substances to the solutions of the dye to be injected. In addition, he believed that staining was improved by the presence of glucose in the dye solution up to a percentage of 2%, and to aid glycolysis he added small quantities of insulin and tartaric acid.

Heller, Thomas & Davenport studied the effects of osmotic pressure, pH, and the presence of dextrose, acetate, pyruvate, or lactate, and agents affecting cell

permeability in solutions of methylene blue used for staining tissues supravitally. They came to the conclusion that the only factors of importance were pH, which should be around 5·6, and the presence of small quantities of acetate and lactate. We have tried all the modifications suggested above, using, however, vital staining methods either by intravenous infusion of the dye or by injecting the dye into the anterior chamber of the eye, and can find no improvement following their use.

II. Silver

We started by using the Bielschowsky-Gros and Gros-Schultze techniques, as described by their originators, and as employed by previous investigators who have worked on the cornea, and we have also tried the Bodian method. However, we were not satisfied with the results we obtained, particularly in comparison with specimens stained with methylene blue. We therefore turned to a silver technique devised by Dr P. Glees, and found this to give much better results; in our opinion it is one of the best silver techniques described. The cornea after careful removal (see p. 175) is fixed for 7 days in 10 % formol saline, then washed for about 20 min. in running tap water. Vertical or tangential frozen sections are then cut and placed in a solution of 10 % silver nitrate dissolved in glass-distilled water. The sections remain in this solution for 12 hr., all light being excluded. The sections are then processed individually. They pass through two changes of 10 % formalin in tap water until no white clouds pass from the sections into the surrounding solution. They are then transferred to the following mixture: one part of 20 % silver nitrate in glass-distilled water and one part of 96 % alcohol. To this mixture is added ammonia 0·880 until the precipitate formed is *just* dissolved. The sections are agitated in this solution for between 30 sec. and 1 min. They are then rapidly taken through two changes of 10 % commercial formalin in tap water until no further white clouds are given off. They are next washed rapidly in two changes of glass-distilled water and placed in 5 % sodium hyposulphite solution, where they are agitated for a few seconds. The sections are finally passed through ascending grades of alcohol, cleared in a mixture of equal parts of carbol xylol and creosote, and mounted in balsam. As in all silver methods the exact time during which a section remains in any particular solution is a matter of trial and error, and, moreover, varies from cornea to cornea. This method has the great advantage that the axis cylinders are impregnated heavily and appear dark black; the Schwann elements appear brown, and the connective tissue only light brown in colour. With the Bielschowsky-Gros method the connective tissue tends to be more heavily impregnated. In all silver methods that we have tried it has been found difficult to impregnate nerve fibres, specifically when they are in close relation to the corneal epithelium, for the epithelial cells themselves are usually as heavily impregnated as the nerve fibres.

III. Gold chloride

We have tried two methods, following Ranvier and Kulchitsky, and found that the former gave somewhat better results.

Ranvier's method we interpreted as follows: The cornea is carefully removed and placed in fresh filtered lemon juice for 15 min. It is then washed rapidly and transferred to 1 % gold chloride for 20 min. Following this it is placed in distilled water,

to which has been added a 'trace' of acetic acid (five drops of glacial acetic acid in 50 c.c. of distilled water), for 1 day; the specimen is then ready for examination. Such preparations show well the general pattern of innervation of the cornea, but they become very dark after a few days. Sections can be made from these preparations by dehydrating, clearing and embedding in wax. The sections do not change colour and have the great advantage that the nerves in relation to the epithelium can be clearly distinguished in contrast to silver sections.

Kulchitsky's method we interpreted as follows: The cornea is first placed in 5 % chloral hydrate for between 1 and 3 hr. It is then quickly rinsed in distilled water and placed in 0·5 % gold chloride in the dark for about 15–20 min., or until the tissue turns yellow. It is then placed in distilled water, to which has been added a 'trace' of acetic acid (5 drops in 50 c.c.), for 24 hr. It is then washed in distilled water, dehydrated, cleared and embedded, and sections are cut. The results we have obtained so far, however, have not been so successful as those prepared by Ranvier's method, for they are somewhat too dark and much gold is precipitated on the surface of the cornea.

IV. Nageotte's method for staining Schwann cells

We carried out the following procedures: The cornea is fixed in 30 % alcohol for about 24 hr. and then transferred to a 1 % solution of nitric acid for a similar period. It is then transferred to 10 % formol in which it remains for 3 days. Frozen sections are then cut and stained in Ehrlich's haematoxylin. This method does not demonstrate the finer histological details of the disposition of the corneal Schwann elements, but is, nevertheless, valuable when used in parallel with other methods.

V. Osmic acid

The cornea after careful removal is fixed in 10 % formalin for 1 week, washed, dehydrated, cleared and embedded in paraffin. Sections are cut and stained in 1 % osmic acid for around 96 hr.; the exact period in osmic acid for optimum results unfortunately varies from cornea to cornea and thus the method of trial and error has to be employed. The specimens are then prepared for mounting in balsam in the usual way. If osmic acid treatment is too prolonged, the whole tissue becomes intensely black, and nerve fibres cannot be distinguished from the surrounding tissues. If the period in osmic acid is too short, the myelin sheaths of the nerve fibres at the limbus are not stained.

To avoid the possibility of dissolving away the myelin in the alcohol stage we also tried cutting frozen sections rather than embedding in wax. The results, however, were no better than those obtained after wax embedding.

VI. Phase-contrast microscopy

We proceed as follows: Thin slices of tissue are cut with a razor from the living corneas of anaesthetized animals or recently dead (3–6 hr.) human subjects. The sections are placed on thin glass slides in physiological saline solution, surmounted with thin glass cover-slips, and examined immediately. In the case of the frog and rat whole corneas can be usefully examined without the necessity of cutting sections. The objectives used were $\frac{2}{3}$ and $\frac{1}{6}$ in. fitted with annular phase plates.

OBSERVATIONS

We have examined in all 259 corneas obtained from the following animals: dogfish, frog, rat, rabbit, guinea-pig, macaque monkey and man. The observations that we shall record are common to all species we have examined and, when possible, have been illustrated by photomicrographs. No attempt will be made in this paper to describe in detail the neural topography of the cornea; this will be done in a subsequent paper.

(1) *The nerves in the living untreated cornea*

These can be distinguished from the tissues around them on examination of the cornea with the standard slit-lamp microscope. They appear as white threads on a grey background. With the magnifications available and the relatively low contrast between the nerve and the surrounding tissues it is not possible to distinguish much detail. Nevertheless, the nerve fibres in the substantia propria can be seen to have a smooth contour, whilst the fibres in the epithelium have a granular or beaded appearance. It is not possible, however, to trace fibres from their origin to their terminations using the standard form of apparatus. We have attempted to photograph corneal nerve fibres under slit-lamp illumination, using a modified optical system in place of the standard binocular microscope, and a light source of increased intrinsic brilliancy, but so far without success.

(2) *The effects of methylene blue on the cornea and corneal sensitivity*

The instillation of methylene blue into the conjunctival sac in man by the method described on p. 173 is followed by a burning sensation which commences 2–3 min. after the drops have been applied. Corneal sensitivity is present throughout the experiment, the lightest touch with cotton-wool or the application of fine hairs causing pain and a corneal reflex, even when at the end of 30 min. all the superficial nerve fibres and their terminals are stained blue. The burning sensation lasts for about 2 hr., at the end of which time a few nerve axis cylinders still remain blue. It is difficult to compare the thresholds to stimulation for eliciting pain or a corneal reflex between the normal and experimental eye by the testing methods commonly employed, but it is sufficient to say that it is not possible to detect any gross difference between the two eyes by this means.

In the rabbit instillation of the dye into the conjunctival sac is not followed by any visible nociceptive response, nor is it possible to detect any gross change in the threshold necessary to elicit the corneal reflex between the normal and stained cornea throughout the experiment by ordinary testing methods.

The general effect on the cornea of instillation of methylene blue into the conjunctival sac in man and in the rabbit is transient. In man the burning sensation referred to above is accompanied by 'injection' of the conjunctiva. After 3 hr. the conjunctival 'injection' recedes, and it is not possible to detect any morphological change in the cornea on slit-lamp examination; moreover, the subject is symptom-free. It is possible to stain the corneal nerve fibres in man repeatedly in this way without either visible deterioration in the quality of the picture seen or ill effects to the cornea itself.

In the rabbit the instillation of dye is followed by some conjunctival 'injection', but this is less obvious than in man, and the staining process can be repeated time and again without any ill effect on the cornea itself or any alterations in the staining of the nerve fibres. Thus the method is applicable in cases where the cornea or its nerve fibres have been subject to experimental procedures.

(3) *Nerves in the living cornea stained with methylene blue*

(*a*) *Following instillation into the conjunctival sac*

The superficial nerve fibres can be clearly distinguished under the corneal microscope, although the deeper nerve trunks and fibres in the substantia propria are not seen. It is possible to examine the cornea so stained under relatively high magnification by substituting a standard microscope for the 'slit-lamp' binocular attachment. Under such conditions the terminations of the nerve fibres in the epithelium can be clearly seen as well as the detailed composition of the more superficial nerve bundles in the substantia propria. The epithelial nerve fibres are characteristically beaded from the moment they become visible after instillation of the dye until they are no longer visible after the lapse of some 2 hr. or more.

Unfortunately, the appearances described, although easy to see, are exceedingly difficult to photograph. Pl. 1, fig. 1, shows a nerve trunk in the substantia propria from the cornea of a rabbit. This should be compared with Pl. 1, fig. 7. In our opinion there is no fundamental difference between them. Pl. 1, fig. 2, is of a beaded nerve fibre in the epithelium of a rabbit's cornea just after piercing Bowman's membrane. This should be compared with Pl. 1, figs. 4 and 8. Again there is no fundamental difference between the two pictures, in our opinion.

The use of the slit lamp for illumination is of no value in such preparations, the nerves appearing just as if they had not been stained.

(*b*) *Following injection into the anterior chamber, or a combination of instillation and injection*

The results obtained are similar save that deep as well as superficial nerve fibres and terminals are also fully stained.

(4) *Nerves in excised corneas stained by injection of methylene blue into the anterior chamber of the eye combined with instillation of the dye into the conjunctival sac*

(*a*) *Examined immediately after removal of the cornea before treatment of any kind*

In Pl. 1, fig. 3, is shown a nerve bundle in the substantia propria of the cornea from a rabbit. Some of the nerve fibres are smooth in outline, others are beaded, but in every case there is a blue thread connecting the beads. No Schwann cell elements are visible, but a few cells in the substantia propria are stained. No organized nerve endings are seen in such preparations.

In Pl. 1, fig. 4, beaded nerve fibres are seen in the superficial layers of the corneal epithelium of the macaque monkey. Again, small threads of stained axis cylinder connect the beads together. The beads are circular in outline and vary somewhat in size.

(*b*) *Examined after treatment as outlined on p.* 177

Pl. 1, fig. 5, is a low-power photomicrograph showing the general pattern of the nerve fibres in the substantia propria of the cornea of a guinea-pig. As in the case of the previous pictures the nerve axis cylinders are seen to be stained deeply, but no Schwann elements are visible. In this case no cells in the substantia propria have taken up the dye.

Pl. 1, fig. 6, shows a nerve bundle in the substantia propria of a rabbit's cornea. This picture should be compared with Pl. 1, fig. 3. In our opinion no fundamental difference can be seen between the pictures presented by either method of preparation.

Pl. 1, fig. 7, shows another similarly stained nerve bundle and is included to show the wealth of fine fibres which can be seen in the cornea of the rabbit. This picture also should be compared with Pl. 1, fig. 3.

Pl. 1, fig. 8, shows fine beaded nerve fibres in the corneal epithelium of the rabbit. This picture should be compared with Pl. 1, fig. 4, and again in our opinion no fundamental difference can be detected between the two pictures. The two photomicrographs in this case came from slightly different positions in the cornea, which accounts for the different arrangement of the nerve fibres.

(*c*) *Examined after variations in the concentration of methylene blue*

The corneas were prepared for examination as described under (*b*). If the concentration of methylene blue injected into the anterior chamber is higher than that recommended, the Schwann nuclei and cytoplasm become stained. In Pl. 1, fig. 9, is shown the result of introducing 0·1 c.c. of a 0·5 % methylene blue solution into the anterior chamber of the eye of a rabbit. Here the Schwann cell elements are deeply stained and it is not possible to distinguish the axis cylinders. The effect of introducing 0·1 c.c. of a 0·25 % methylene blue solution into the anterior chamber of the eye of a rabbit is shown in Pl. 1, fig. 10. In this case the axis cylinders are well stained, but Schwann nuclei can also be seen. Fine processes of the Schwann cytoplasm also take up the dye and may be confused with nerve fibres.

(5) *Nerves in the cornea after intravenous infusion of methylene blue*

The corneas were excised and prepared for microscopic examination after 'fixation' of the dye with molybdate and dehydration with ethyl alcohol. In Pl. 2, fig. 11, nerve fibres are seen to be well stained in the region of the limbus, and, whilst a few fibres can be seen passing towards the centre of the cornea, they are faintly stained and most of the finer fibres are not stained at all.

Pl. 2, fig. 12, shows what can be seen in the centre of the cornea using this method, and should be compared with Pl. 1, fig. 8. The irregular passage of the nerves in Pl. 2, fig. 12, is the result of distortion following dehydration in ethyl alcohol. This picture is in sharp contrast to the appearances in Pl. 1, fig. 8.

(6) *Nerves in the cornea following immersion in methylene blue*

The technique used was that according to Heller *et al.*, described above. In Pl. 2, figs. 13, 14, the axis cylinders are seen to be poorly differentiated and in many cases appear to be discontinuous. The tissues of the nerve sheath, on the other hand, have

taken up the dye in considerable concentration. These figures should be compared with the previous pictures, particularly Pl. 1, figs. 3, 6; the contrast is striking, and clearly the nerve fibres depicted by this method bear no relation at all to their appearance in the living state.

(7) *Nerves in the cornea after silver impregnation*

We used the method of Glees described above, and found that silver impregnation is useful for studying the nerve fibres in the substantia propria. It is difficult to distinguish fine nerve fibres in the superficial layers of the epithelium by this method, for the epithelial cells themselves usually become deeply impregnated when the nerve fibres in the substantia propria are well differentiated. On the other hand, when the section is less heavily impregnated, only a few nerve fibres can be distinguished. Pl. 2, fig. 15, shows a nerve bundle in the substantia propria of a rabbit and should be compared with Pl. 1, figs. 3, 6. Pl. 2, fig. 16, shows a single nerve fibre from the same preparation, and Pl. 2, fig. 17, is also from the same preparation showing neuro-fibrillae in a single nerve fibre. In Pl. 2, fig. 16, the Schwann elements are lightly stained, and in some cases it is very difficult to distinguish between nerve axis cylinders and the more deeply impregnated parts of the Schwann elements. In Pl. 2, figs. 18, 19, are shown fine nerve fibres in close relation to the corneal epithelium of the dogfish.

These illustrations of silver preparations of nerve fibres are the best that we could obtain, and clearly the fibres do not have the same appearance as they do in the living cornea.

(8) *Nerves in the cornea after gold chloride impregnation*

This method gives very beautiful pictures of the pattern of corneal innervation when viewed as a whole preparation; for instance, as shown in Pl. 3, fig. 20 (rabbit's cornea), which should be compared with Pl. 1, fig. 5. However, details of the finer ramifications of nerve fibres are not well shown, for the gold is precipitated unevenly on the axis cylinders of the nerve fibres and on other tissues, and this makes the certain tracing of a single nerve fibre from a nerve trunk to its final destination impossible. Pl. 3, fig. 21 (rabbit's cornea), illustrates this point. On the other hand, when sections of the corneal epithelium are prepared by this method, the manner of distribution of the fine beaded fibres is very well demonstrated. This is illustrated in Pl. 3, fig. 22 (cornea from macaque monkey). Some fine precipitation can be seen in the epithelium and on the surface of the cornea. Comparison of Pl. 3, figs. 20–22, with methylene-blue stained preparations (Pl. 1, figs. 3–6) shows that the method of impregnation with gold chloride gives a good idea of the general neural pattern of the cornea, but cannot be used for the determination of the finer details of the neuro-histological arrangements.

(9) *Artefacts*

During the course of our work we have been impressed by the great care necessary in the preparation of specimens to avoid introducing artefacts. Fortunately, the possibility of examining the living stained cornea has enabled us to determine the various ways in which such artefacts are produced. If, in the course of preparation

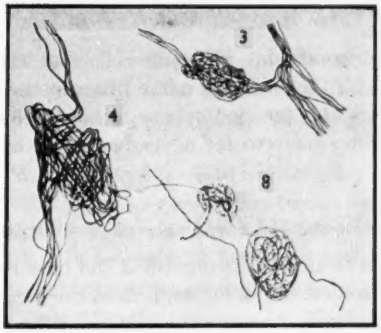
of the cornea for microscopical examination, attempts are made to flatten the tissue forcibly at any stage, distortion of a proportion of the nerve fibres always occurs. Some fibres may be compressed, in which case they appear to pursue a wavy course and their axis cylinders have no regular diameter. Other fibres may be stretched, when the axis cylinder is either attenuated or ruptured. In the latter case the methylene blue-stained axis cylinders appear as an irregular series of disconnected beads of varying diameter; this is illustrated in Pl. 3, fig. 23. Again, the axis cylinder may take the form of a series of disconnected segments. Occasionally such a segment appears to end in the form of a club and may well be mistaken for a terminal end-organ (Pl. 3, fig. 24). If excessive pressure is used, some of the fluid portion of the axis cylinder may be extruded; nerve fibres may then appear to end in the form of a terminal spray which may well be mistaken for a group of Krause end-bulbs.

The cornea may be more readily flattened by making two or three radial incisions into it. However, if this is done directly after removal, and particularly if the cutting instrument is blunt, some of the axis cylinders are stretched before being severed. Since they are elastic, they may spring back and form a complicated coil in which some of the stained axis cylinder substance may or may not be extruded into the meshes of the coil. Such appearances may also be mistaken for complicated nerve end-organs. However, since these structures are found only in close proximity to the incision, the cause of their appearance is in no doubt. This is well illustrated in Pl. 3, fig. 25, which shows five such endings in close proximity to a radial incision into the cornea, and Pl. 3, figs. 26–28, show such artefacts under a high power of the microscope. We have included a number of them for comparison with facsimile copies of drawings of specialized endings pictured as occurring in the cornea by Dogiel (1891) and Egorow (1934) (Text-fig. 1).

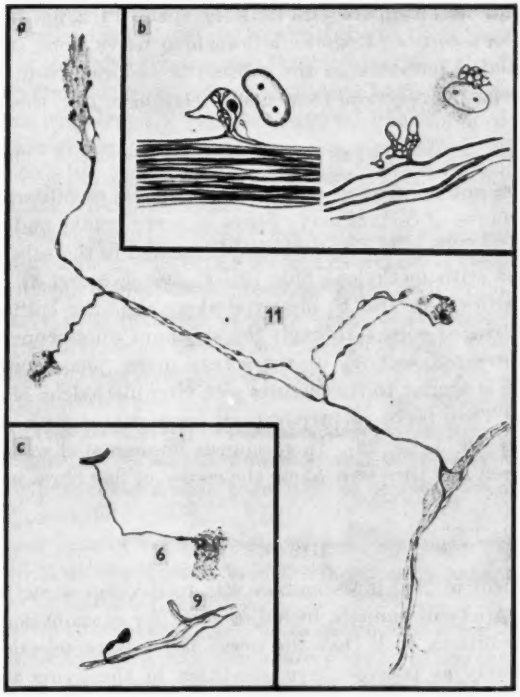
The second type of artefact often confused with specialized nerve endings is the result of over-staining by methylene blue or over-impregnation with gold or silver. Such treatment stains or impregnates cells in the substantia propria, including their fine protoplasmic processes, which look very like nerve fibres and may be in close relation to them. Pl. 4, figs. 29–32, show such artefacts clearly in preparations somewhat over-stained by methylene blue. In Text-fig. 2 are further facsimile copies of drawings of specialized corneal nerve terminals from the publications of Dogiel (1891), Egorow (1934), and Boeke (1935), for comparison.

(10) *Schwann cells and nuclei*

Using Nageotte's method, we have seen Schwann cells in the substantia propria, but never in the corneal epithelium (Pl. 4, fig. 34). Schwann cells and their nuclei can also be demonstrated by over-staining with methylene blue (Pl. 1, figs. 9, 10), but again only in the substantia propria of the cornea. Silver staining can also be used to demonstrate the Schwann cells around nerve fibres in the substantia propria (Pl. 2, fig. 16). The pictures illustrating Schwann cells following silver impregnation and over-staining by methylene blue all show that the Schwann cytoplasm has a fibrillary appearance under the conditions which have been employed. They should be compared with Pl. 1, figs. 5–7, and Pl. 2, fig. 15, in which the nerve axis cylinders alone are selectively demonstrated.



Text-fig. 1. Showing facsimile copies of three illustrations of 'specialized nerve terminals' in the cornea. Drawings labelled 3 and 4 are from a paper by Egorow (1934) and described as 'Nervenknäuelapparat'; they are from the guinea-pig's cornea stained with methylene blue. The drawing labelled 8 is from a paper by Dogiel (1891). It is described as an 'Endknäuel' from a human cornea stained with methylene blue.



Text-fig. 2. Showing facsimile copies of five illustrations of 'specialized nerve terminals' in the cornea. Drawing *a*, labelled 11, is from a paper by Dogiel (1891). It is described as an 'Endplättchen' and is from a human cornea stained with methylene blue. The drawings labelled *b* are from a paper by Boeke (1935). They are described as collateral endings and are from silver preparations of a falcon's cornea. The drawings labelled *c* are from a paper by Egorow (1934). The drawing labelled 6 is described as an 'Endplättchen' and the drawing labelled 9 is described as a reticulated end-apparatus. Both are of methylene blue-stained preparations from the guinea-pig.

Under phase-contrast conditions Schwann cells and their nuclei can be seen. They are found only along the course of nerve fibres in the substantia propria, but not all the nerve fibres in this position appear to have Schwann cells in relation to them. Beaded nerve fibres have so far never been seen to have Schwann cells in relation to them.

(11) *Corneal nerve fibres under the phase contrast microscope*

Single nerve fibres can be clearly distinguished and their relationship to the tissues surrounding them determined. Pl. 4, fig. 35, is from the cornea of a rabbit and shows a single nerve fibre apparently lying on the surface of the cytoplasm of a Schwann cell, the nucleus of which can be clearly seen. The fibre in focus is part of a small nerve bundle, the remaining fibres of which are out of focus. Pl. 4, fig. 36, is from a different part of the same preparation and is included to emphasize that the nerve fibre in focus is again lying on the surface of the Schwann cell cytoplasm. Pl. 4, fig. 37, shows a beaded nerve fibre in the epithelium of a rabbit's cornea. Note the 'end bead' and compare with Pl. 1, fig. 4, and Pl. 2, fig. 18. Pl. 4, fig. 38, is also from a rabbit's cornea. It shows a branching nerve fibre, smooth in outline, giving rise to beaded terminals in the substantia propria. Note that the smooth preterminal fibre has no Schwann elements in relation to it in this position.

(12) *Beaded nerve fibres*

So far we have not referred in detail to the *regular* moniliform beading which appears on the course of certain nerve fibres in nerve trunks and constantly along the finer terminals, both in the corneal epithelium and in the substantia propria, in specimens stained with methylene blue (Pl. 1, figs. 4, 5 and 8). These swellings, beads, or varicosities, can also be observed along the finer epithelial nerve fibres in the living untreated cornea through the slit-lamp microscope. They are again seen in fresh untreated sections of the cornea under phase-contrast conditions. Their appearance is similar to the pictures seen after methylene blue staining (Pl. 4, figs. 37 and 38). They occur in preparations impregnated with gold, particularly in the epithelium (Pl. 3, fig. 22). In specimens impregnated with silver, however, they are rarely seen and then only along the course of fine fibres in close relation to the epithelium.

DISCUSSION

Our major problem in this investigation was to develop a method of preparing corneas from a variety of animals, including man, for examination under standard microscopical conditions such that the nerve fibres were selectively stained and resembled as closely as possible their condition in the living tissue. From our observations it will be clear that the technique which, in our hands, has come closest to fulfilling these conditions is methylene blue staining of the axis cylinder. We shall now proceed to discuss the various advantages and disadvantages of this method. The advantages are:

(1) Methylene blue can be used to stain the axis cylinders of nerve fibres selectively in the living state. Observations made with both the slit-lamp and phase-contrast

microscopes have shown that selective staining of nerve axis cylinders with methylene blue does not of itself lead to any detectable change from the living state in the morphology of the nerve fibres or their terminals, neither does it alter their relationship to surrounding structures.

(2) It is possible to control the degree of staining by examination under the corneal microscope, and thus eventually to prepare tissues for microscopical examination in which the nerve axis cylinders have been selectively stained.

(3) The corneal nerves may be selectively stained and examined repeatedly at chosen intervals following experimental procedures such as keratotomy or section of nerve fibres that may innervate the cornea.

(4) There is no evidence that methylene blue, used in the concentrations necessary to stain selectively all the axis cylinders present, irreversibly damages the nerve fibres concerned or has any adverse effect on the other tissues of the cornea.

(5) Following selective staining the cornea can be removed for critical microscopical examination before it is subjected to further treatment of any kind.

(6) Whole corneas in which the nerve axis cylinders have been stained selectively can be examined under high magnifications without sections having to be cut. The complete course of the axis cylinders can therefore be determined.

(7) By altering the concentration of methylene blue it is possible to stain, in addition to the axis cylinders, the cytoplasm and nuclei of the corneal Schwann cells. Such concentrations also stain the histiocytes and certain other cellular elements in the cornea. It is thus possible to determine to some extent the relation of the nerve axis cylinder to these cellular elements.

(8) It is possible to prepare permanent preparations of whole corneas for ordinary microscopic examination in which the nerve axis cylinders have been specifically stained with methylene blue without distortion or the production of artefacts.

(9) Corneal nerve axis cylinders seen in specimens specifically stained with methylene blue resemble those seen in specimens following the use of the silver-impregnation technique described above.

The disadvantages of this method are:

(1) Although methylene blue can be used as a specific stain for nerve axis cylinders, the technique is such that to achieve perfect results the greatest care is necessary; it thus demands experience and some knowledge of neuro-histology before it can be used as a routine method.

(2) In the present state of our knowledge, specific nerve axis cylinder staining by methylene blue without the production of artefacts can be achieved only by introducing the dye to *living tissues in situ*.

(3) It has so far not been found possible to use routine specific counter-stains in combination with, or following, methylene blue staining of the axis cylinder without either destroying its specificity or introducing artefacts.

We shall now compare and contrast the results obtained following methylene blue staining with those following the impregnation and other methods we have used.

The gold chloride techniques are undoubtedly valuable, for they impregnate corneal nerve fibres with a considerable degree of specificity. However, it is clear from our observations of methylene blue-stained living nerve fibres that the

techniques cannot be used to evaluate fine neuro-histological details, and so far we have not found it possible to prepare specimens free from artefacts. That is not to say that the method could not be improved so that it could yield a more specific picture, free from artefacts.

Fortunately, the silver-impregnation method that we use, and for which Dr Glees is responsible, is capable of a considerable measure of control, and we have been able to use it to produce sections free from gross artefacts and pictures of certain nerve fibres which are comparable to those seen after methylene blue staining. So far, however, we have not been able to demonstrate by this method all the nerve fibres that exist in the cornea. From our brief experience with the method, however, it seems to us that this technique might well be still further improved and eventually used to obtain pictures of nerve fibres and their terminals from fixed corneas which will be strictly comparable with those obtained after staining with methylene blue. In view of the frequent necessity of using fixed material, and the widespread use of silver-impregnation methods to demonstrate the neural elements in a variety of tissues, we feel that it is of the greatest importance that attempts should be made to improve silver methods by comparison with specific staining methods, for, if successful, it might then be possible to assess the pictures so obtained in relation to the morphology of living nerve fibres from all parts of the body.

Since medullated nerve fibres are found only at the extreme edge of the normal cornea, myelin stains have not proved of much value in this investigation. The literature, however, suggests that in certain pathological conditions in man nerve fibres throughout the cornea may be medullated (Schornstein, 1934; Koke & Braley, 1940). The evidence for this was their appearance in the untreated cornea under the slit-lamp microscope.

We found that Nageotte's method of staining the Schwann cells is useful only in confirming observations on specimens over-stained by methylene blue.

The picture seen of nerve fibres and their terminals in the untreated cornea under the phase-contrast microscope resembles in detail that seen after selective staining of the axis cylinder by methylene blue. In addition, it is also possible by this method to determine precisely the relation of the nerve fibres and their terminals to the tissues surrounding them. The use of phase-contrast microscopy, however, is of value only when examining relatively thin sections or whole preparations from small corneas; thus, unlike methylene blue staining, it cannot readily be used to build up a total picture of corneal innervation in animals with corneas larger than, for instance, the white rat's. We have found that its greatest value has been to confirm and expand the observations made using methylene blue staining.

The ease with which the neural elements can be damaged when preparing a cornea for microscopical examination is a matter which, we believe, has wide implications. It is true that the cornea is a delicate tissue and that the nerve fibres therein are not protected by tough fibrous sheaths as they are elsewhere; nevertheless, we have reason to believe that artefacts can be produced only too easily in the neural elements of other tissues prepared for microscopical examination. The neuro-physiologist will undoubtedly agree that in the handling of nervous tissues the greatest care is necessary to prevent damage so extensive as to render quantitative measurements impossible. It is not surprising, therefore, that equal care is necessary in the pre-

paration of specimens for microscopical examination. It might be thought that a method involving staining of the axis cylinder with vital dye (which, although it may affect the physiological properties of the nerve fibre to some extent, certainly does not abolish conduction) might be particularly subject to distortion in the course of preparation for microscopical examination. This is probably true, for the dye appears to be in a fluid state in the mammalian axon (Woollard, Weddell & Harpman, 1940), and Young (1936) has shown that when the Giant Squid axon is cut across, axoplasm flows from the cut end. Nevertheless, we have shown that other techniques also introduce artefacts, and that this problem has not been sufficiently taken into account by those describing neuro-histological pictures, particularly when their relationship to function is inferred. In the paper by Weddell & Glees (1941) using various histological techniques, even following fixation by intravenous perfusion of the fixative, pictures were given of normal cutaneous nerve fibres and terminals in the rabbit's ear. It is now clear to us that most of the pictures given were to some extent distorted, and that sufficient care had not been taken in removing the skin from the ear cartilage, nor, in the course of preparation, to avoid the introduction of artefacts. Weddell & Glees also made reference to the existence of a number of nerve fibres exhibiting signs of degenerative changes, such as follow axonal interruption, in all the normal specimens they examined. It is clear that, although some of the fibres referred to may indeed have been degenerating, the whole problem needs reinvestigation in the light of our present observations. We have included one photomicrograph of a methylene blue-stained piece of skin from the rabbit's ear (Pl. 4, fig. 33) to illustrate this point. That this preparation had been mishandled is only too obvious on comparison with the other photomicrographs given of normal corneal nerves; yet, in fact, the skin was carefully prepared using routine methods. In connexion with this point it is worth mentioning a paper by Langworthy & Ortega (1943) on the innervation of the iris. They used methylene blue staining and show pictures of nerve fibres which are in the form of gross beads without interconnexions (cf. Pl. 3, fig. 23). Such fibres could hardly be expected to exist in this form in the living state (let alone conduct impulses), and yet no mention is made in the publication of the possibility that distortion might have occurred in the course of the preparation of these tissues for microscopical examination.

We have never seen any specialized nerve end-organs in carefully prepared specimens of the cornea stained with methylene blue in a concentration sufficient to stain the axis cylinder specifically, nor have we seen any under the phase-contrast microscope, even though the profusion of nerve fibres and beaded terminals seen under both conditions was greater than has been described by previous authors. Moreover, the ease with which artefacts resembling nerve end-organs can be produced leads us to believe that most of the larger terminals described in the literature as being present in the cornea proper were due to mishandling of the tissue in some way. Some of the illustrations given by Dogiel, Egorow and Boeke fall, we believe, into this category, as can be judged by Text-fig. 1. The description by Yokomatsu (1931), quoted by Reiser, of over twenty types of specialized nerve end-formations in the cornea must also be regarded with suspicion, as must the description by Goto-Yotaro (1935), also quoted by Reiser: 'so existieren in der Hornhaut nach Goto Udonge-, Weidenästchen-, Federbusch-, Chrysanthemum-, Warabi-, und spargel-

blätterähnliche Endigungen. Neben diesen seien noch knäuelartige und vogelfuss-ähnliche Endapparate und freiendigende Nervenfasern in der Hornhaut vorhanden.'

In addition to the specialized endings which we believe to be artefacts resulting from mishandling of the tissue a number of writers have described the existence of fine nerve fibrils and other end-formations, the existence of which we have not been able to confirm, either under the phase-contrast microscope or when methylene blue has been used in concentrations that stain the nerve axis cylinders specifically. From our observations it is clear that such demonstrations are the result of non-specific staining methods, for we ourselves have been able to render them visible in this manner (see Pl. 4, figs. 29–32 inclusive and Text-fig. 2).

Prolonged and careful examination of the living cornea by slit-lamp microscopy shows that the fine superficial nerve fibres are beaded. This observation is in confirmation of the statements and illustrations of Vogt (1921). Nevertheless, we have so far failed to get enough illumination to photograph them. The demonstration by phase-contrast microscopy of their existence along the course of corneal nerve fibres has, however, settled the issue as to whether or not they are produced by the action of methylene blue itself. The demonstration that they can be seen in the untreated cornea under the phase-contrast microscope is important in view of some of the statements that appear in the literature; for instance, Ciaccio (1863) and Petermoeller (1869) describe the existence of beaded nerve fibres in the substantia propria of the cornea after treating it with acetic acid, and do not refer to the beading as an artefact. Cohnheim (1867) states that the varicosities along the course of the nerve fibres in the corneal epithelium are morphological entities. Ranvier (1881) and Apathy (1897) state their belief that the beads are not part of the axis cylinder but due to impregnation of a circum-fibrillary oily substance. Virchow (1910) mentions beading along corneal nerve fibres, but believes this to be a post-mortem change. He is confirmed in this conclusion by Engelmann's (1867) statement that beads appear in the frog's cornea only when the tissue is allowed to dry, and can be avoided if the cornea is kept in a moist chamber during examination. Boeke (1925, 1935), using silver methods, states his belief that the beading which can be seen along corneal nerve fibres is an artefact due to formol fixation.

The earliest signs of degeneration following interruption of a nerve fibre are irregularities of the axon, quickly followed by fragmentation and the formation of beads. Clearly, before early degenerative changes can be recognized with certainty, it is necessary to exclude the possibility that mishandling of the tissue has not itself introduced artefacts which might be mistaken for degenerative changes (Pl. 3, fig. 23). Thus at the moment it is only possible in the majority of cases to evaluate the existing condition by a comparison of the pictures given by normal and suspected tissues which have been treated in exactly the same manner.

The introduction of phase-contrast microscopy for the examination of corneal nerve fibres has enabled certain other controversial questions to be settled. In our review of the literature a quotation from a paper by Ciaccio, written as early as 1863, was given. Ciaccio states clearly that, following immersion in acetic acid, the corneal corpuscles are seen to have processes resembling nerve fibres and may be mistaken for them unless great care is taken. His observations were disputed following the use of more complicated methods. Nevertheless, phase-contrast pictures have shown that he was correct in his observations.

The fact that it is possible to stain living nerve axis cylinders with methylene blue selectively without abolishing nerve conduction raises the question as to the nature of the process involved. At the moment there are no experimental data to allow us to do more than make suggestions. We have assumed that the nerve axon contains substances with special affinity for methylene blue or an associated substance. The reaction appears likely to be enzymatic in nature in view of the minute quantities in dye involved in the process. Unfortunately, the 'methylene blue' (Merck) that we find giving consistent results is known to be a complex dye, and we are not even certain that it is the tetra-methyl thionin contained in it that is responsible for the reaction. This may be the reason why Nakajima was never able to stain nerve fibres successively from day to day using Grübler's dye; indeed, he states that he believed the mixture he used was toxic. In view of this we are now injecting solutions of pure methylene blue and other related dyes in pure form into the anterior chamber of the eyes of white rabbits and comparing the results obtained with those following the use of the Merck compound. It seems to us that if we can obtain data related to the process involved, we may well be able to devise methods of using methylene blue as a generally applicable nerve axis cylinder stain, far surpassing those at present available, and at the same time increase our knowledge of nerve axon metabolism. It is also clear that either new methods or modifications of old methods of fixation, dehydration and clearing will have to be devised if we are going to be in a position to supply the neuro-physiologist with neuro-anatomical data upon which to evaluate his observations.

SUMMARY

1. A method of preparing whole corneas from various animals for neuro-histological examination is described. It involves: (*a*) the use of 'methylene blue' which stains the living nerve axis cylinders specifically, uniformly and constantly throughout the cornea; and (*b*) a method of 'after treatment' which allows corneas so stained to be excised, mounted and preserved as preparations for examination by ordinary microscopical methods without any detectable morphological change.

2. Pictures of living nerve fibres obtained by the use of the slit-lamp microscope and those seen under the phase-contrast microscope have been shown to be comparable with those obtained as described above.

3. Corneas can be stained as described at repeated intervals following experimental procedures.

4. The beads seen along the course of nerve fibres in corneas so prepared are not artefacts.

5. No specialized nerve end-formations in the cornea proper from man, the macaque monkey, the guinea-pig and the rabbit have been seen. The specialized end-formations described in the literature can be produced by the technical methods employed and are, in our opinion, artefacts.

6. Very strict attention to technical details is necessary to avoid distortion and artefact formation.

7. A silver impregnation method has been described which gives pictures resembling closely those seen in the living state.

8. The mode of action of methylene blue and its possible development as a neuro-histological stain of wider application has been discussed.

9. It is suggested that until new methods of fixation and dehydration are developed for use after methylene blue staining, it is not possible to equate accurately neuro-histological findings in tissues other than the cornea with neuro-physiological observations.

This investigation was made possible by a grant from the Rockefeller Foundation, to whom we wish to express our gratitude. Our thanks are also due to Dr Joseph E. Hawkins, Jr., of the Merck Institute, Rahway, New Jersey, U.S.A., for supplying us with the samples of methylene blue used in our experiments.

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EXPLANATION OF PLATES

Plate 1

All figures are from methylene blue preparations.

Fig. 1. Showing a nerve bundle in the substantia propria of the cornea of a living rabbit photographed *in situ* 30 min. after the injection of dye into the anterior chamber of the eye. The photograph was taken by reflected light from a flash of 300 msec. duration. Compare fig. 7. (×140.)

Fig. 2. Showing beaded nerve fibres in the corneal epithelium of a living rabbit photographed *in situ* 30 min. after injection of the dye. Procedure as for fig. 1. Compare fig. 8. (×570.)

Fig. 3. Showing a nerve bundle in the substantia propria of the cornea of a rabbit after injection of dye into the anterior chamber of the eye and instillation of dye into the conjunctival sac 20 min. previously. In this case the photograph was taken immediately after removal of the cornea from the eye, before treatment of any kind. Compare figs. 1, 6 and 7. (×360.)

Fig. 4. Showing beaded nerve terminals in the corneal epithelium of the macaque monkey. The cornea was stained, excised and photographed in the same way as that shown in fig. 3. Note the continuous nerve loops. (×690.)

Fig. 5. From the cornea of a guinea-pig showing the general pattern of innervation and the way in which the nerve fibres pierce Bowman's membrane. The photograph in this case was from a permanent preparation made as outlined on p. 177. (×90.)

Fig. 6. Showing a nerve bundle from the substantia propria of a rabbit's cornea also prepared as outlined on p. 177. Compare fig. 3. (×350.)

Fig. 7. From a slightly different position in the same preparation as depicted in fig. 6, and should be compared with fig. 3. (×380.)

Fig. 8. Also from the same preparation as shown in fig. 6 and shows beaded nerve fibres in the epithelium. It should be compared with fig. 4. (×380.)

Fig. 9. From the substantia propria of the cornea of a rabbit in which the concentration of the dye was in excess of that required for specific staining of the nerve axis cylinder. (×110.)

Fig. 10. From a similar preparation to that shown in fig. 9, save that the concentration of the dye was only just above optimum for specific staining of the axis cylinder. It shows a nerve bundle in the substantia propria. (×620.)

Plate 2

Fig. 11. From a whole preparation of the cornea of a rabbit after intravenous infusion of methylene blue and preparation by 'fixation' in ammonium molybdate and dehydration in ethyl alcohol. (×45.)

Fig. 12. From a whole preparation, but comes from the centre of a rabbit's cornea following intravenous infusion of methylene blue and preparation by 'fixation' in ammonium molybdate and dehydration in ethyl alcohol. Compare fig. 8. (×300.)

Fig. 13. From a whole preparation of the cornea of a rabbit following excision and immersion in a solution of methylene blue. Compare figs. 5 and 6. (×75.)

Fig. 14. Also from a whole preparation of a rabbit's cornea following immersion in a solution of methylene blue. Compare figs. 3, 6 and 9. (×130.)

Fig. 15. Showing a nerve bundle in the substantia propria in a section from a rabbit's cornea impregnated with silver. Compare figs. 3 and 6. (×370.)

Fig. 16. From the same preparation as fig. 15. Note the impregnation of the Schwann elements. (×320.)

Fig. 17. From the same preparation as fig. 15. Note the 'neurofibrillae'. (×750.)

Figs. 18, 19. From sections of the cornea of a dogfish showing fine beaded nerve fibres impregnated with silver in close relation to the corneal epithelium. (×600.)

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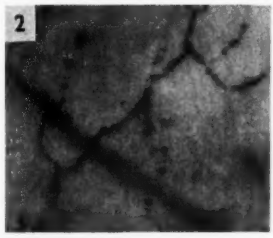
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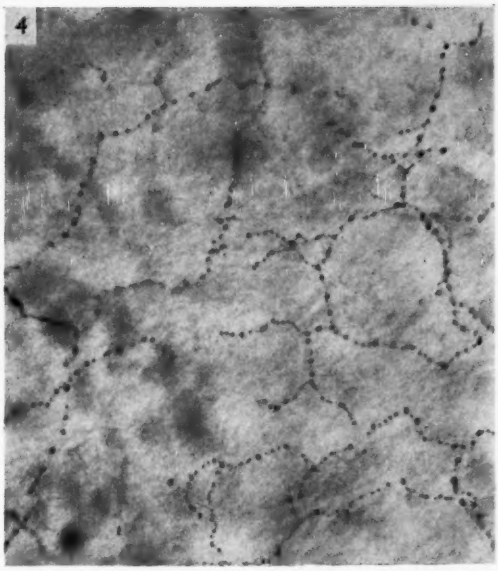
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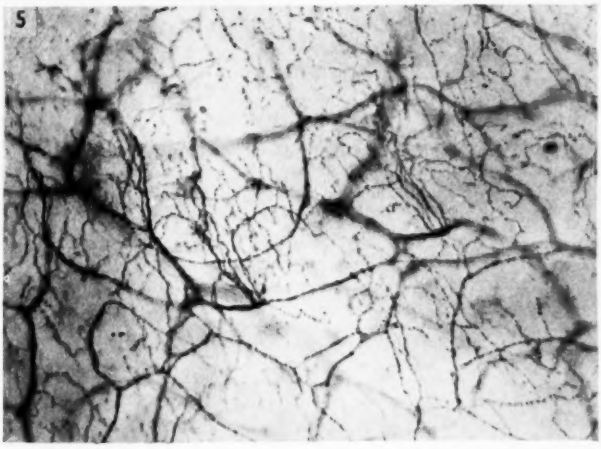
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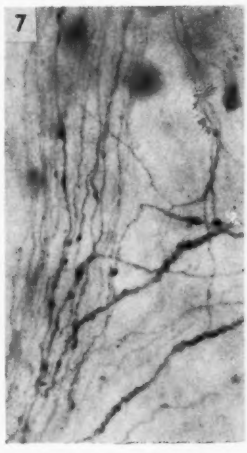
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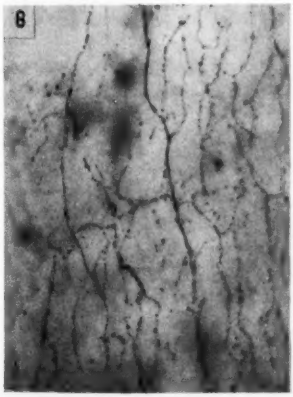
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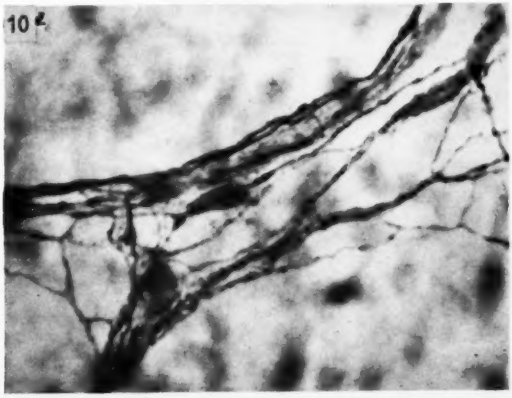
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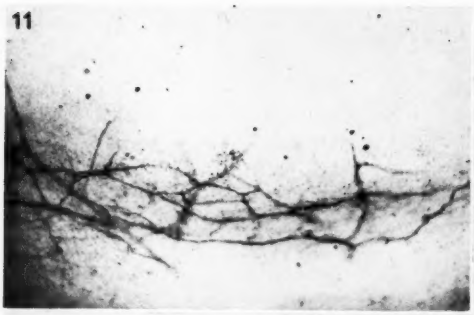
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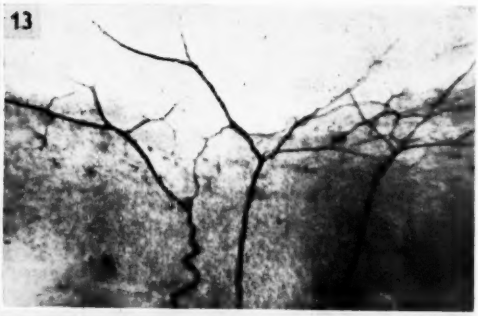
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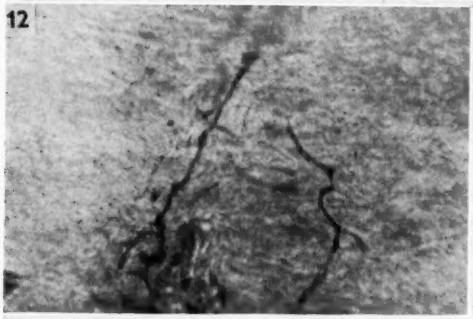
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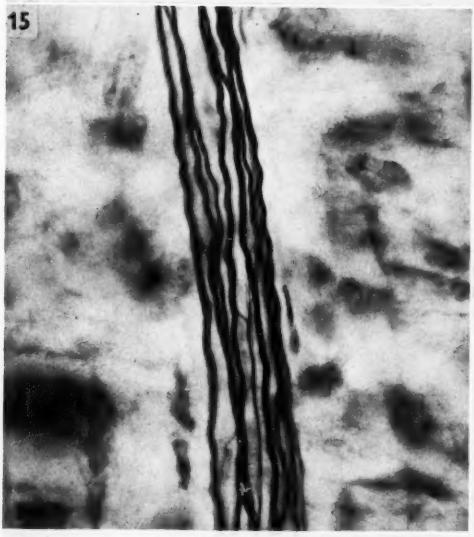
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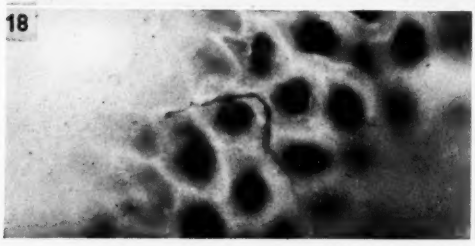
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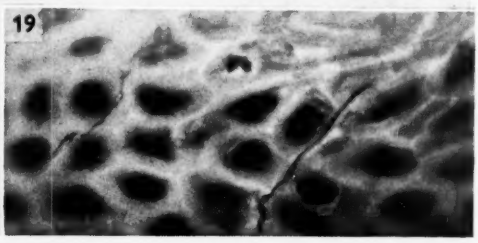
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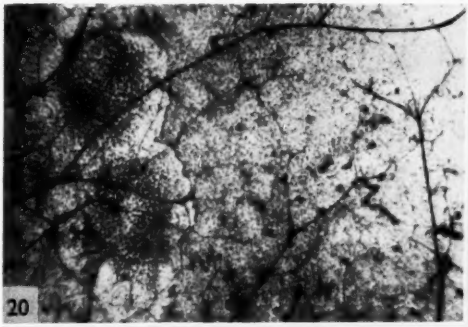
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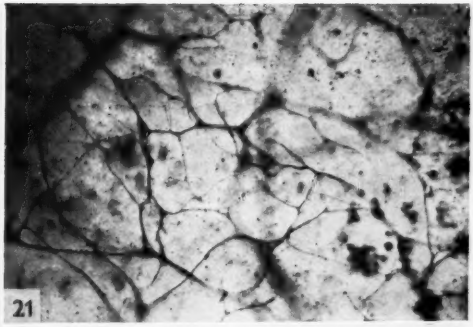
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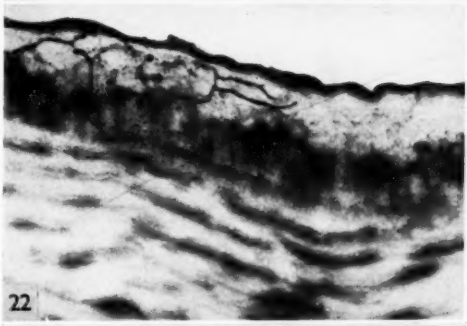
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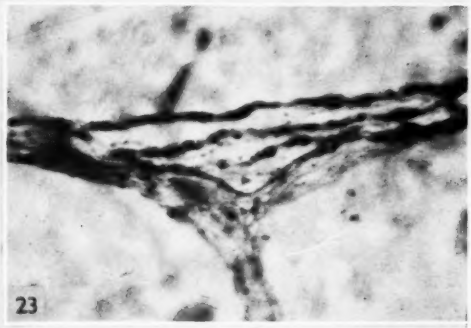
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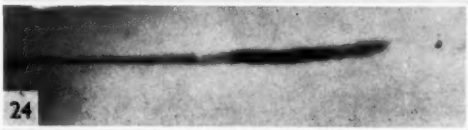
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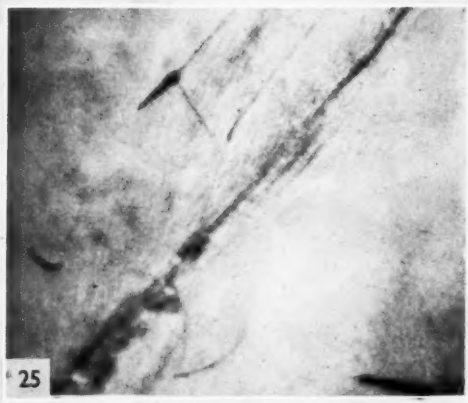
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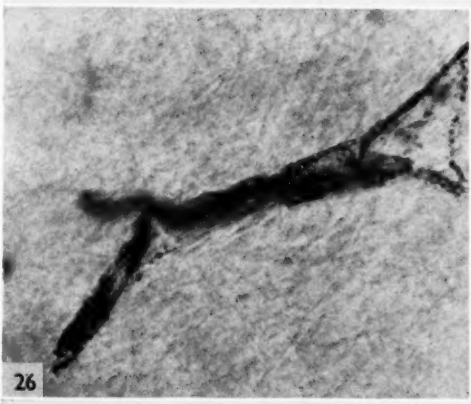
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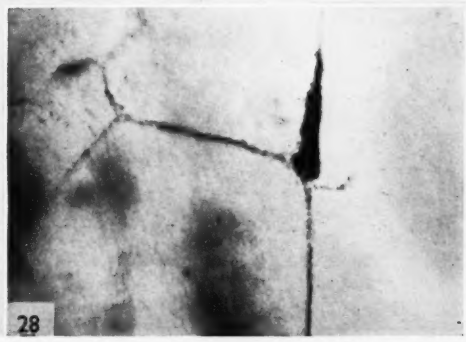
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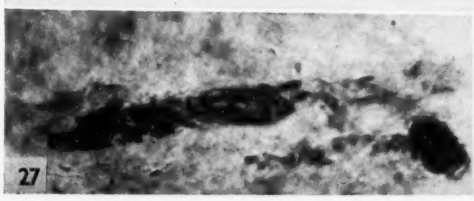
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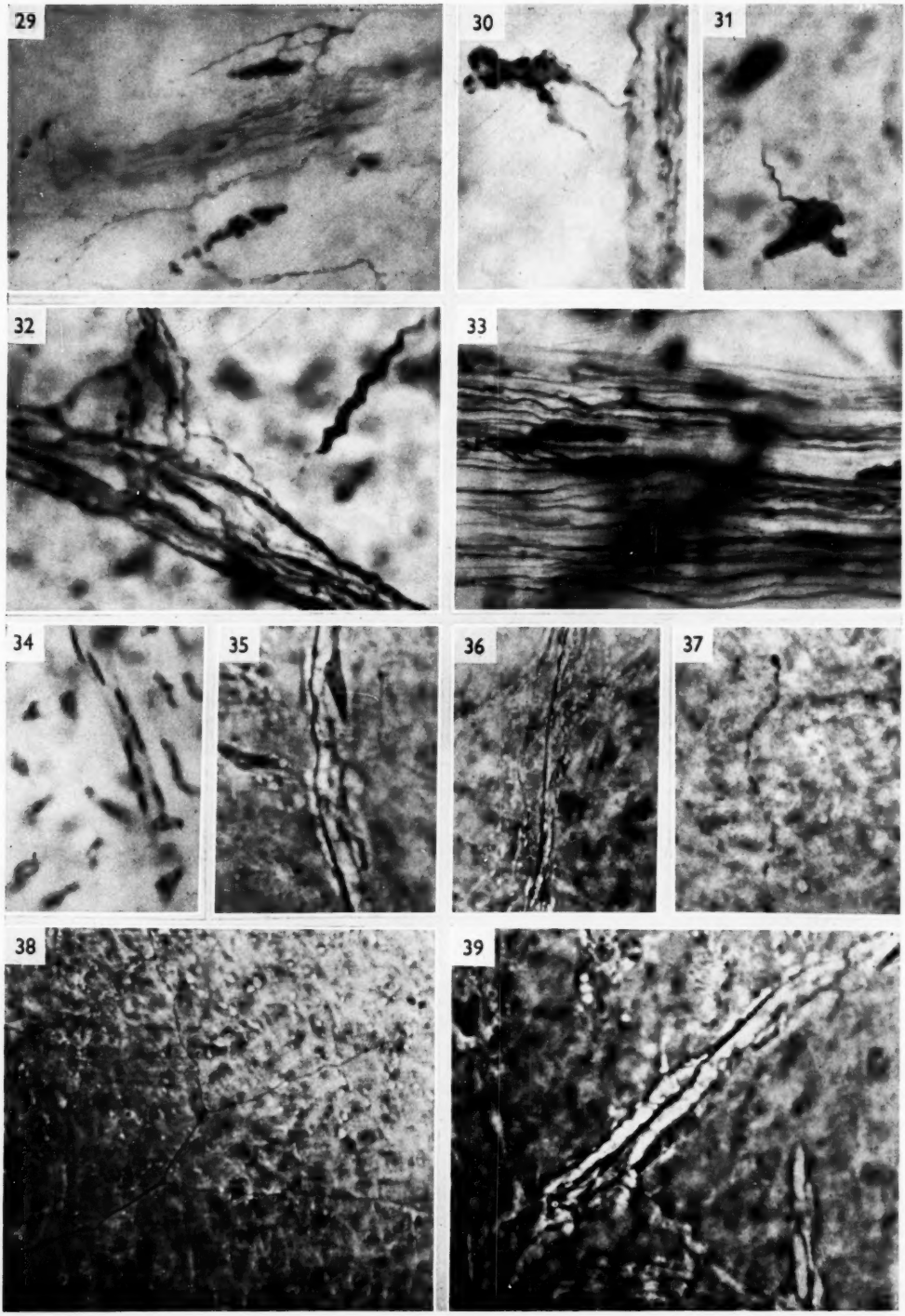
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PLATE 3

Fig. 20. From a whole preparation of the cornea of a rabbit after gold impregnation of the nerve fibres. (×80.)

Fig. 21. Also from a whole preparation of the cornea of a rabbit after gold impregnation of the nerves. Compare with fig. 5. (×110.)

Fig. 22 From a section of a macaque monkey's cornea after gold impregnation of the nerves. (×450.)

Fig. 23. From a whole preparation of the cornea of a rabbit after the injection of methylene blue into the anterior chamber of the eye. In this case the preparation had been forcibly compressed in the course of preparation. (×380.)

Fig. 24. Also from a whole preparation of a rabbit's cornea following injection of methylene blue into the anterior chamber of the eye and which had been roughly handled in the course of preparation. (×300.)

Fig. 25. From the cornea of a rabbit following injection of methylene blue into the anterior chamber of the eye. Artefacts which have been referred to in the literature as 'specialized endings' are seen on either side of an incision made into the cornea using blunt scissors. (×90.)

Figs. 26–28. Showing artefacts or so-called 'specialized endings' similar to those seen in fig. 25 under a higher magnification. These photomicrographs should be compared with the 'endings' depicted in Text-fig. 1. (Fig. 26, ×270; fig. 27, ×290; fig. 28, ×280.)

PLATE 4

Figs. 29–32. Showing the kind of artefacts which can be produced in whole preparations of the rabbit's cornea merely by increasing the concentration of methylene blue injected into the anterior chamber of the eye. The tissues were otherwise prepared as outlined on p. 177. These photomicrographs should be compared with the 'endings' depicted in Text-fig. 2. (Fig. 29, ×200; fig. 30, ×500; fig. 31, ×500; fig. 32, ×450.)

Fig. 33. From skin stripped from the ear cartilage of an albino rabbit following local injection of 0·01% methylene blue 30 min. previously. 'Fixation' was in ammonium molybdate and dehydration by ethyl alcohol. (×260.)

Fig. 34. From a section showing Schwann cells in the substantia propria of the cornea of a rabbit following its preparation by Nageotte's method. (×90.)

Fig. 35. From an excised, but otherwise untreated, cornea of a rabbit under the phase-contrast microscope. It shows a nerve fibre and associated Schwann elements in the substantia propria. (×600.)

Fig. 36. From the same preparation as depicted in Fig. 35, but in a different position. It shows much the same thing. (×500.)

Fig. 37. Showing a beaded nerve fibre in the corneal epithelium from the same preparation as that depicted in fig. 35. Compare figs. 4 and 18. (×750.)

Fig. 38. Also from the untreated excised cornea of a rabbit under the phase-contrast microscope. It shows a branching preterminal nerve fibre, smooth in outline, giving rise to beaded terminals in the substantia propria. Note that the nerve fibre has no Schwann elements in relation to it. (×560.)

Fig. 39. From the same preparation depicted in fig. 38. It shows a nerve bundle under phase-contrast conditions and emphasizes the difficulty of interpretations in such cases. (×620.)

CYTOLOGICAL AND HISTOCHEMICAL VARIATIONS IN THE ADRENAL CORTEX OF THE ALBINO RAT

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In a previous paper (Harrison & Cain, 1947) considerable variation in the distribution of lipoids in the adrenal cortex of albino rats was described, and it was suggested that this variation might be cyclic. In the present paper further observations on these variations are reported, and they are described histologically and cytologically.

MATERIAL AND METHODS

Albino rats, inbred for at least ten generations, from the colony in the Department of Human Anatomy, were used, as in our previous investigation. Most of the animals were killed by chloroform; in all cases the adrenals were immediately removed and placed in fixative. Killing by chloroform was chosen for its rapidity. The appearances to be described did not differ from those in the members of litters killed by decapitation, coal-gas or nembutal overdosage (see later). Baker's acid haematein method (Baker, 1946, 1947; Cain, 1947*c*) was used, as in our previous investigation, for phospholipines. Sudan Black (Lison, 1936; Cain, 1947*a*) and Nile Blue (Cain, 1947*b*, 1948*c*) were used on frozen sections of adrenals fixed in formal calcium (Baker, 1944). The plasmal reaction was carried out in a modified form (Cain, 1949), which is described later. Since methods employing osmium tetroxide are not suitable for demonstrating the Golgi apparatus, Aoyama's technique was used, and for mitochondria the acid fuchsin-methyl blue technique (Cain, 1948*a*) after fixation in Helly's fluid. In addition, to facilitate comparison of our results with those of Hoerr (1931, 1936*a*,*b*), most of the techniques given above were also applied to guinea-pig adrenals, on which Hoerr worked.

RESULTS

General description of the variations

In each case, with the 127 rats examined, one adrenal was used for the acid haematein test, the other for one of the other techniques given above. The patterns of variation seen were those described in our previous paper. Consequently, the results previously obtained with acid haematein are considered together with those now reported for the first time, making a total of 154 rats. To avoid wearisome repetition, the general pattern of the variations will be described first, so that individual adrenals can be characterized by 'stage' numbers instead of lengthy descriptions.

The variations are defined with respect to the zona fasciculata, and are most clearly seen by means of the acid haematein method. Briefly, the cells of the z. fasciculata may be filled with lipoid droplets, particularly in the outermost part of this zone, or completely discharged of such droplets, there being intermediate

stages. The active, secretory stage is characterized by an abundance of phospholipine demonstrable by the acid haematein test. For convenience in description the variations are divided into nine stages which are arranged so that any one stage resembles much more those described just before and just after it than any of the other stages. That such an arrangement is possible is strong evidence for some sort of sequence in the variations (but not, of course, for a regular periodicity):

Stage I. Considerable phospholipine is seen only in a few cells of the outermost z. fasciculata (except for very small quantities which show a characteristic distribution, described below, in all the cells of the cortex).

Stage II. The outermost quarter of the z. fasciculata is heavily loaded with phospholipine (and stains an intense blue or blue-black with acid haematein).

Stage III. The outer half of the z. fasciculata is intensely positive.

Stage IV. The outer two-thirds are positive.

Stage V. The whole z. fasciculata is heavily positive.

Stage VI. The z. fasciculata becomes less positive, usually very patchily.

Stage VII. Large areas are nearly negative to acid haematein. These areas may be principally in the outer regions, principally in the inner, or irregularly distributed.

Stage VIII. Very few cells are heavily positive. This stage usually resembles stage I, but the cytological appearances are different.

Stage IX. No cells in the z. fasciculata show more than a few scattered bodies positive to acid haematein.

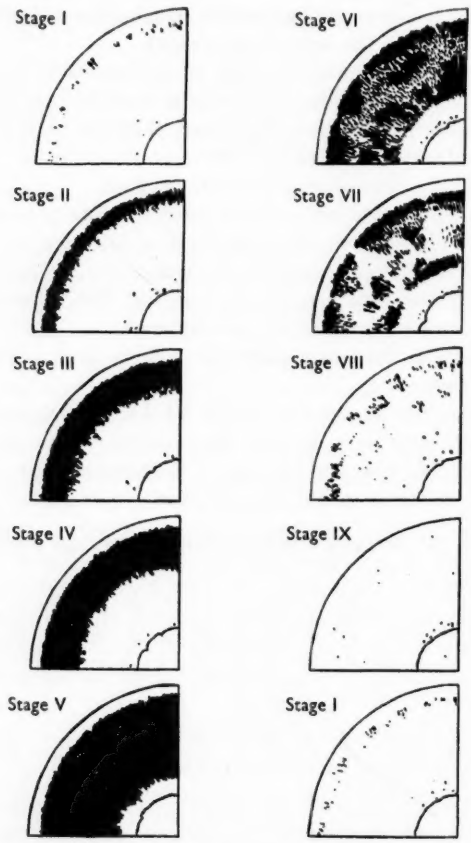
Stages I–V are regarded as secretory stages, VI–IX as discharge stages. Stage IX (complete discharge) may be a resting phase. These stages are shown in Pl. 1, figs. 1–8, and Pl. 2, figs. 1–4, and illustrated diagrammatically in Text-fig. 1. The behaviour of the z. glomerulosa is discussed later.

Table 1. *Adrenals examined classified by stage numbers*

	I	I–II	II	II–III	III	III–IV	IV	IV–V	V	V–VI	VI	VI–VII	VII	VII–VIII	VIII	VIII–IX	IX	IX–I	Totals
Immature males	5	5	12	—	5	3	2	1	2	1	—	—	—	—	2	—	4	4	46
Mature males	—	—	2	—	—	2	2	—	—	—	—	—	—	—	—	—	—	—	6
Immature males 11 weeks after orchidectomy	—	—	1	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	2
Immature females	7	8	27	2	5	4	2	—	—	1	2	—	2	2	8	2	4	3	79
Mature females, oestrous stage undetermined	2	—	—	—	—	—	—	—	—	—	—	—	1	—	—	1	1	1	6
Mature females in oestrus	1	—	—	—	—	—	—	—	—	—	—	2	—	1	—	—	—	—	4
Mature females in dioestrus	1	—	—	—	—	—	—	—	—	—	1	—	—	1	1	—	—	—	4
Pregnant females	—	—	1	—	—	—	—	—	—	—	—	—	1	—	1	—	—	—	3
Immature females 4 weeks after ovariectomy	—	—	—	—	1	—	—	—	—	1	—	—	—	—	—	—	—	—	2
Immature females 11 weeks after ovariectomy	—	—	1	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	2
Totals	16	13	44	2	12	9	6	1	2	3	4	2	4	4	12	3	9	8	154

The results obtained on the adrenals examined are summarized in Table 1. In determining the stage number of a given adrenal, several sections must be examined, because it often happens that different parts of the cortex are not at the same stage. For example, a given section which is principally at stage III may show a few isolated regions at stage II, or some parts of the z. fasciculata may be at stage IV.

In such cases the adrenal as a whole is assigned to stage III. The stage numbers shown in the table have been determined in this way. It is rare for one adrenal to show greater variation than in the examples just given, and adrenals in stages IX, I, II and III usually show very little. From Table 1 it will be seen that the early secretory stages (I–IV) were most frequently observed; the next most frequent were stages in the late discharge phase (VIII and IX).



Text-fig. 1. Diagram of the stages described in the text. Each drawing represents one-quarter of a transverse section through the centre of an adrenal. The annuli of which each is composed represent, from the outside inwards, z. glomerulosa, z. fasciculata (shaded to some extent), z. reticularis and medulla. The shading represents heavy or medium concentrations of phospholipine.

Cytology of the variations

(*a*) *Mitochondria and Golgi apparatus*

Examination of one adrenal of a rat by the acid haematein method and of the other by some different technique allows the behaviour of cell organelles and in-

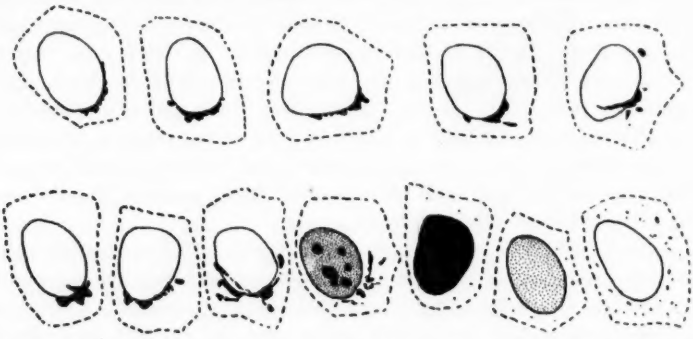
clusions to be studied in relation to histochemical variations. The two adrenals normally present the same histological and cytological appearances (Tobin & Whitehead, 1942).

We find that the mitochondria and Golgi apparatus show, by means of the standard techniques, no certain signs of alteration with the different histochemical appearances. In all cells of the cortex the mitochondria appear after the acid fuchsin-methyl blue method as very numerous small spheres, intensely fuchsinophil. In secretory cells, as described by other workers (e.g. Hoerr, 1931, 1936*b*), they are found scattered in the cytoplasm between the droplets of lipoid. In non-secretory cells they are much more abundant, and form a great part of the cytoplasm. In dying cells they become irregular and may clump together. Occasionally, especially in the z. reticularis, a slight variation in size may be seen, the diameter of some being increased by about one-third. This may be seen also in secretory cells of the outer z. fasciculata, but under no circumstances have any forms transitional between normal mitochondria and lipoid droplets been seen, and all our observations confirm Hoerr's conclusion that such forms are due entirely to bad fixation.

Hoerr states (1936*b*) that silver methods for the Golgi apparatus fail to produce more than a few scattered granules in the guinea-pig cortex. In the rat we find that Aoyama's method is very reliable, except that the z. glomerulosa is often unsatisfactorily impregnated. The rest of the cortex, and the medulla, impregnate very satisfactorily. The apparatus appears as an irregular cap, applied to one side of the nucleus, often with irregular prolongations in various directions, as described by Bennett (1940) in the cat. (The orientation described by him is not clear in the rat.) In the guinea-pig cortex we find, in agreement with Hoerr, that Aoyama's method usually fails, but in a few patches of cells a Golgi apparatus very similar to that in the rat cortex can be seen. It is noticeable that in the rat the apparatus in the z. glomerulosa and outer z. fasciculata is usually very compact, but becomes progressively more and more diffuse and fragmented as one passes inwards to the medulla. But this phenomenon has no relation to stage appearances and may vary enormously in degree from one part of a section to the next. In some extreme cases we find that the general appearance is as just described, but in other parts, as one passes inwards, a diffuse Golgi apparatus is seen in the outer regions, then none at all, then a region in which the nuclei are impregnated, then again a region in which nothing can be seen (Text-fig. 2). These phenomena are more frequent in large (adult) adrenals than in small ones, and it is considered, therefore, that the usual apparent fragmentation of the Golgi apparatus in the inner region of the cortex is merely the first of a series of effects due to uneven fixation, and cannot be taken to indicate a change in the structure of the Golgi apparatus. Aoyama's fixative and other fluids for silver-impregnation do not preserve the cytoplasm of cells very adequately. Bourne (1934*a*), using da Fano's technique, found a hypertrophy of the Golgi apparatus in *Trichosurus vulpecula*, *Petaurus breviceps*, and immature albino rats, which he associates with the production of lipoid droplets. While from his figures this may be the state of affairs in the opossum and flying phalanger, we have been unable to confirm it in the rat by using only silver methods. However, there are certain bodies described fully below which are shown by Sudan Black, and correspond so closely to the spheroid complexes described by Baker (1944) and

others (Thomas, 1947, 1948; Cain, 1947*a*, 1948*b*) that they seem to be a part of the Golgi apparatus. Their presence in the cells does point to a hypertrophy of part of the Golgi apparatus, but they are not shown by the silver method, and moreover, cannot be shown by Sudan Black after fixation and impregnation as for Aoyama's technique.

Very rarely the Golgi apparatus in the z. reticularis is visible after acid haematein, and shows a positive reaction for phospholipine.



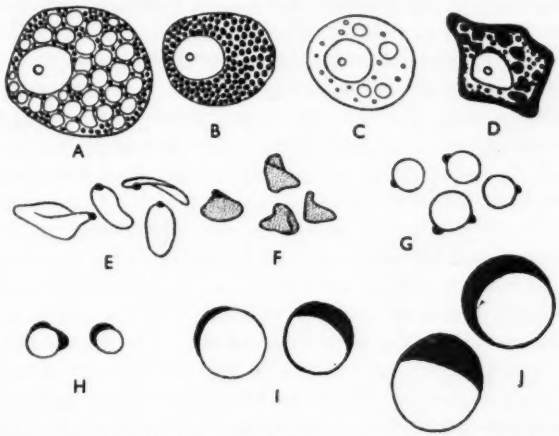
Text-fig. 2. Drawings of cells of the cortex as seen after Aoyama's technique. Cells from the outermost z. fasciculata on the left, from the innermost on the right. In the upper row the Golgi apparatus appears compact nearly throughout; in the lower row gradations between the compact form, the diffuse form, and a failure of impregnation are seen. Both rows are from the same section.

(*b*) *Acid haematein* (Text-fig. 3)

Although the mitochondria show no changes in their morphology, their reactions appear to vary considerably. As mentioned above they are always fuchsinophil (after fixation in Helly and post-chroming). The changes in their reactions to the acid haematein test, however, are responsible for most of the differences between the various stages. The objects seen in cortical cells after acid haematein can be classified into three groups: mitochondria, the discharge bodies (a miscellaneous group of granules and droplets), and spheroid complexes.

(i) *Mitochondria.* In all cells heavily positive to acid haematein (that is, in all cells shown in black in Text-fig. 1) the mitochondria are the bodies which stain. In cells of the outer z. fasciculata which are loaded with lipoid droplets (the so-called spongiocytes) they appear as blue-black spheres scattered in the cytoplasm between the droplets which do not stain. The appearance is exactly that seen in the same region in a standard mitochondrial preparation. In cells of the same region in which lipoid droplets have not yet accumulated, they are, of course, packed much more closely together. The pyridine extraction control test shows nothing in cortex or medulla except some erythrocytes, all resting nuclei, and the chromosomes of dividing nuclei. Consequently, the positive mitochondria must contain very large quantities of phospholipine. Many mitochondria in other tissues have been found positive to acid haematein (Baker, 1946; Cain, 1947*c*), and the presence of phospholipines in them has been demonstrated biochemically by several workers, especially

Claude (1941), but in the rat adrenal cortex the mitochondria are positive only in certain regions. In all cells lying between the inner edge of the heavily positive zone and the medulla (that is, in most of the z. fasciculata in stage I, the inner three-quarters in stage II, half in stage III, quarter in stage IV, and in all of it in stage IX) the mitochondria are negative. The only exceptions are a few mitochondria in cells nearest the heavily positive zone, that is, in cells in a transitional state, and many if not all of the mitochondria in dead or dying cells. These cells are common in some sections in the z. reticularis, and may occur in addition scattered in the z. fasciculata. They are intensely positive to acid haematein. The less altered cells show distinct mitochondria, many swollen or irregular, tending to clump together. The others are usually blue-black throughout, the whole cytoplasm being positive.



Text-fig. 3. Bodies seen in the cytoplasm with acid haematein. A, B: cells with and without lipoid droplets (unstained) showing mitochondria. C: cell in transitional state (secretory to discharge) showing faint mitochondria. D: dying cell, with mitochondria and large positive droplets, probably mitochondrial aggregates. E, F: discharge bodies. E, composite bodies usually found in the middle and outer z. fasciculata; F, bodies characteristic of the inner z. fasciculata and z. reticularis. An intermediate form is shown between the two groups. G: clear droplets with granules attached, perhaps part of the spheroid complex series. H–J: spheroid complexes arranged in increasing size. Those at J are specially characteristic of the z. reticularis.

(ii) *Discharge bodies.* Those cells which are not in the secretory phase, and therefore not *heavily* positive to acid haematein, may show a large variety of bodies, one group of which, the spheroid complexes, is discussed below. The others comprise very palely staining globules; irregular leaflets and granules, each usually (perhaps always) with a much more darkly staining granule attached; and rather darkly staining granules of various sizes, sometimes appearing like aggregations of smaller bodies. Their usual distribution is as follows:

In cells transitional from the secretory to the discharge state the mitochondria often appear very faintly. The globules of lipoid which load the cytoplasm, completely unstained in secretory cells, may also appear, very faintly stained, as the pale globules mentioned above. In cells rather nearer the medulla, the mitochondria and lipoid globules are not usually seen. Instead, in each cell, there are numerous small bodies

(far fewer in number than the mitochondria or lipoid droplets) which vary considerably in shape. Some appear to be granular, nearly spherical or very irregular; others are leaflets, usually ovoid or obovoid. In general, as one goes from the outer z. fasciculata to the z. reticularis, they become progressively reduced in number, and more and more darkly staining. Sometimes the darker bodies appear as small clumps of minute granules, but this is not usual, and there is no evidence whatever that the reduction in numbers is due to aggregation. The more lightly staining bodies towards the middle and outer part of the z. fasciculata are usually composite. Attached to an edge or elsewhere on the surface is a minute granule, extremely positive, rather like a mitochondrion but smaller. Occasionally, in cells nearer the medulla, intermediate forms may be seen in which the main part of the composite body is more positive.

(iii) *Spheroid complexes.* In addition to the above bodies, or more rarely instead of them, composite bodies of an entirely different nature may be seen. These consist of completely unstained spheres (usually appearing glossy-white against the faintly yellow-brown cytoplasm) either completely invested by an outer layer of varying thickness, or with a large or small cap, or a granule (seldom two) attached. The attached or investing body is very positive to acid haematein. These are illustrated in Text-fig. 3 H–J. Occasionally, rather large colourless spheres with one or two minute granules attached have been observed (Text-fig. 3 G). Some of these have more the appearance of bloated composite discharge bodies rather than small members of the spheroid complex series; their relationship is obscure.

Large spheroid complexes have been found in the z. reticularis of nearly all the mature rats examined, and occur quite often in the z. fasciculata as well. In immature rats they are far less frequent and are nearly always confined to the innermost z. fasciculata (which corresponds to the z. reticularis in the adult). Very small spheroids have on occasion been found in other parts of the z. fasciculata as well. The cells of the middle and outer z. fasciculata are invariably crowded with positive bodies (either mitochondria or discharge bodies of various sorts), but it is possible that a few minute spheroid complexes may be present in all cells (except perhaps the secretory cells). In mature animals the complexes are as a rule much larger and can then often be seen in all discharge cells. They do not occur in dying cells.

(*c*) *Sudan Black*

The most conspicuous objects in any section coloured with Sudan Black are the lipoid droplets in the cells. These colour intensely black. Hoerr's contention (1936*a*, *b*) that in any one cell the droplets are all the same size is fully confirmed by our observations for all cells in the secretory phase. In discharge cells and dying cells they may vary considerably in size, but one single large droplet filling the cell, as described by earlier workers (reviewed by Hoerr, 1931, 1936*a*, *b*), was never seen. The cytoplasm (except for that of dead or dying cells) is only feebly sudanophil, so that the lipoid droplets are shown very clearly. The mitochondria colour only very feebly, and are not usually distinguishable as such. The various forms of discharge bodies are strongly coloured, appearing only as irregular black opaque objects. Lipoid droplets, coloured faintly by acid haematein, are also black. In a very few cells colourless crystals may be seen, often partly included in a lipoid

droplet. These appear to be crystals of cholesterol or its esters. In the guinea-pig they are far more abundant (Hoerr, 1936*b*).

The large spheroid complexes appear very clearly in Sudan Black preparations. The internum is completely colourless, as before, the attached or investing bodies black. When the whole body is small, it is often hidden by lipoid droplets, or the intensity of coloration of the investing externum may cause the internum to be hidden, so that it appears merely as a small lipoid droplet. Small spheroid complexes are usually easily recognizable in the z. reticularis or inner z. fasciculata.

Rarely, in a few cells of a few sections only, other appearances are seen, not comparable with anything shown by acid haematein. In some acid haematein sections a few mitochondria in the outer z. fasciculata appear as rods of a length four or five times their diameter. In one Sudan Black preparation (of an immature female, stage III) in some cells of the inner z. fasciculata irregular bent rods with irregular swellings are seen. These do not appear to correspond at all to the mitochondrial rods since they are much longer and more irregular (Text-fig. 4A). In three preparations (a mature male, stage IV, a female in oestrus, stage VII–VIII, and a female in dioestrus, stage I–II) there can be seen on the nucleus, in some cells, caps which in optical section appear to have lenticular sudanophil walls, and an uncoloured internum (Text-fig. 4B).

Text-fig. 4. A, irregular rod-like bodies, and B, nuclear caps in optical section, both shown by Sudan Black.

Both these sets of bodies are strongly reminiscent of the Golgi apparatus as seen in Aoyama preparations, the latter set resembling the compact cap-like apparatus, the former the much more diffuse form. But in general, no trace of the Golgi apparatus as seen in Aoyama preparations is visible in acid haematein or Sudan Black preparations.

(*d*) *Sudan IV*

With this colorant, which is considerably less intense than Sudan Black, only the larger lipoid droplets can be seen.

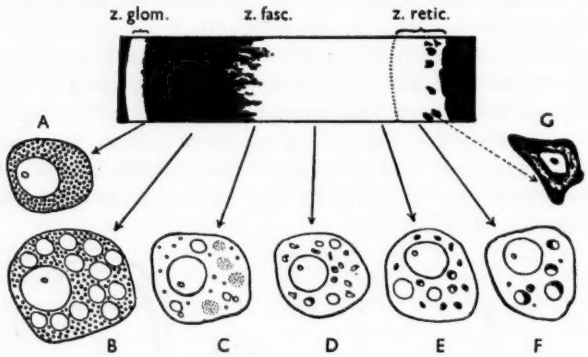
(*e*) *Nile Blue*

With Nile Blue, only the large lipoid droplets are coloured red. Very often they take up the oxazone only slightly, and as the cytoplasm usually stains heavily with the blue dye, their red colour is easily hidden. In many sections no red can be seen

at all, and when, as occasionally happens, the globules appear white, this cannot be due to masking. Quite often the globules themselves are stained blue. The significance of colorations with Nile Blue is discussed below.

(*f*) *Appearances with the modified plasmal technique*

The cytoplasm of most cells of the z. fasciculata is positive with this technique, those near the outer edge of the zone usually being most intensely coloured. The innermost z. fasciculata and the z. reticularis are uncoloured. The lipoid droplets are completely negative. This result is in contradiction to that obtained by Verne (1928), but the technique which he used shows only a *pseudo*-plasmal reaction (Cain, 1949).



Text-fig. 5. Diagram of the cytoplasmic bodies in an adrenal at stage II. A, B, cells in secretory phase; C–E, cells in discharge phase. A: cell with mitochondria positive to acid haematein. B: cell as A but now loaded with lipoid droplets. C: cell with very faint mitochondria and some lipoid droplets faintly positive to acid haematein; transitional between B and D. D: cell with numerous weakly positive composite discharge bodies, some negative lipoid droplets, and a few small spheroid complexes. E: cell with medium positive discharge bodies, not obviously composite, otherwise as D. F: cell from the z. reticularis with large spheroid complexes and small very positive granules. G: a dying cell.

Correlation of cytological appearances with histochemical variations

The direct correlation that can be made between the histochemical variations and the appearances described above is that mitochondria heavily positive to acid haematein, usually accompanied in the same cell by numerous regular lipoid droplets, are the bodies by whose changes in distribution the stages can be recognized. They are responsible for the intensely positive reaction in certain cells upon which the stages described above are based. They appear to be mutually exclusive to all other bodies, except some of the smallest spheroid complexes. The discharge bodies and the medium or large spheroid complexes are characteristic of cells nearer the medulla than those with intensely positive mitochondria, that is, to the areas of the z. fasciculata (and z. reticularis) unshaded in Text-fig. 1. In general, the composite discharge bodies (with granules attached) are characteristic of the outer discharge cells, the simpler and more positive granules of the inner cells. In nearly all the immature rats examined there was no z. reticularis; in the mature animals it was large and easily recognized. Its characteristic appearance is merely that of the

innermost z. fasciculata cells, often exaggerated. The largest spheroids, occasionally seen in a few of the innermost z. fasciculata cells in immature animals, are usually abundant in it, and it often contains dead or dying cells (although these may occur scattered throughout the cortex). It may be regarded as a continuation and exaggeration of the inner z. fasciculata. These relations are summarized in Text-fig. 5. The proportions of the various forms of discharge body vary considerably in different adrenals.

Behaviour of the zona glomerulosa and sudanophobe zone

(*a*) *Zona glomerulosa*

The z. glomerulosa shows various marked differences from the z. fasciculata. The two zones agree in that no variations are evident in the mitochondria and Golgi apparatus in standard preparations. The Golgi apparatus in the z. glomerulosa is usually a rather compact mass closely applied to the nucleus, as in the outer z. fasciculata. The mitochondria rather frequently give the impression of being very short rods rather than spheres, in acid haematein preparations. With acid fuchsin they appear to resemble those of the z. fasciculata but to be slightly smaller, and fewer.

In acid haematein preparations the z. glomerulosa may show principally mitochondria, in which case the cells greatly resemble those of the outer z. fasciculata, but an equal intensity of staining in each cell is seldom attained. The lipoid droplets are unstained. When few or no mitochondria are shown, then, as with the z. fasciculata, the lipoid droplets tend to stain, and quite often are more deeply stained than in the z. fasciculata. These droplets and the mitochondria are the only objects seen in the z. glomerulosa, except that in one section only bodies resembling the composite discharge bodies were observed. The spheroid complexes were never seen in z. glomerulosa cells. Care must be taken in examining acid haematein preparations of the z. glomerulosa, as it may appear darkly stained under the low power through the presence of very numerous blood corpuscles.

The lipoid droplets of the z. glomerulosa are more numerous in each cell than in the z. fasciculata, and consequently tend to deform one another and appear like rows of peas in a pod. As a consequence the z. glomerulosa is often the darkest zone in sections coloured with Sudan Black, so that these preparations are not the easiest to work with when determining stages. The z. glomerulosa is seldom found to contain very few lipoid droplets, and in this it contrasts markedly with the z. fasciculata, which, as already described, varies considerably. Mitochondria positive to acid haematein were observed in the z. glomerulosa principally in adrenals at stages II–VI. With several exceptions, they were not seen in any quantity in adrenals at stages VII–IX and I. This might indicate secretory changes in the z. glomerulosa corresponding to, but out of phase with, those of the z. fasciculata. If so, then there is a great difference in the discharge phase which in the z. glomerulosa is far less abrupt, and shows few or no composite bodies of any sort in the cells. From the cytological appearance it would seem that the cells of the z. fasciculata discharge their lipoid droplets rather rapidly and completely, whereas those of the z. glomerulosa probably discharge them slowly and continuously.

With Nile Blue the same appearances are seen as in the z. fasciculata, but it

should be noted that when some of the droplets are coloured red in a section, those of the z. glomerulosa are coloured as a rule far more deeply than the others. This points to a difference in their physical condition.

With the modified plasmal technique the z. glomerulosa, like the z. fasciculata, shows a positive result throughout the cytoplasm. The lipoid droplets are not coloured.

(*b*) *Sudanophobe zone*

This zone, recognized by many workers, is, as its name indicates, distinguished by its comparative or absolute inability to take up Sudan colorants. In 123 out of the 154 rats examined, the corresponding zone was clearly distinguishable in acid haematein preparations; when Sudan Black preparations were available for comparison the zone was nearly always present.

In acid haematein preparations the sudanophobe zone appears as a more lightly stained band of cells between the z. glomerulosa and the z. fasciculata. The cytological appearances are those of the adjacent z. glomerulosa, but weaker. Thus if the z. glomerulosa contains darkly staining mitochondria, the sudanophobe zone will show either the same but far fewer, or the same but fewer and less intensely positive. When both mitochondria and lipoid droplets are seen in the z. glomerulosa, the sudanophobe zone usually shows only a few droplets. In four sections only composite discharge bodies, or bodies resembling them, were seen in the sudanophobe zone, and on one occasion, small spheroid complexes.

Usually if one examines cells in a series starting with the innermost sudanophobe cells and progressing to the outermost cells of the z. glomerulosa, the change in the cytological appearances is continuous. The innermost sudanophobe cells have every appearance of being young z. glomerulosa cells and of gradually passing into fully secreting z. glomerulosa cells just underneath the capsule. The sudanophobe cells are nearly always much smaller than the z. glomerulosa cells, but the nuclei are the same size. Consequently, over the same area, they show far more nuclear material which is constantly negative to acid haematein and Sudan Black, so that even though their various granules and droplets stain as deeply as do those of the z. glomerulosa, the *general* effect is always of reduced intensity of staining or colouring. The boundary between the sudanophobe zone and the outer z. fasciculata, on the other hand, when the sudanophobe zone is clearly visible, is a sharp one, although a few transitional cells here and there may generally be seen. There is no smooth continuous series, however.

In the 31 rats out of 154 in which the sudanophobe zone appeared to be absent, examination showed that it was still recognizable (although not sudanophobe) and that there were several possible reasons for its disappearance. In a few cases the zone was recognizable with acid haematein but not with Sudan Black. In these the acid haematein-positive bodies in the cells were few, but the lipoid droplets many, so that with Sudan Black it was merged with the z. glomerulosa. In other cases, in which the same effect was obtained with both techniques, again it was merged with the z. glomerulosa. In most of these cases the outer z. fasciculata was strikingly different (though containing much less or much more lipoid than the z. glomerulosa), so that the inner limit of the sudanophobe zone was easily seen. In a few cases

(seven out of thirty-one) it had disappeared since it had merged with the outer z. fasciculata in appearance, while remaining distinct from the z. glomerulosa. In these the z. glomerulosa appeared remarkably narrow.

The distribution of adrenals apparently without sudanophobe zones is shown in Table 2. It will be seen that, in proportion to adrenals with an obvious sudanophobe zone, most are in stages VI–VIII, and in mature animals, but a few occur in all stages.

Table 2. *Presence and apparent absence of the sudanophobe zone*

	Present		'Absent'		
Stage	Immature rats	Mature rats	Immature rats	Mature rats	Totals
I	26	1	2 (1) — (1)	3 (2) — (1)	32
II	41	5	2 (1) — (1)	— — — —	48
III	16	3	1 (1) — —	1 (1) — —	21
IV	4	2	1 — (1) —	— — — —	7
V	3	—	1 (1) — —	1 (1) — —	5
VI	—	3	2 — (2) —	1 — — (1)	6
VII	3	1	1 (1) — —	3 (3) — —	8
VIII	5	1	7 (4) (2) (1)	2 (2) — —	15
IX	9	—	1 — — (1)	2 (1) — (1)	12
Totals	107	16	18	13	154

The figures in parentheses in the 'absent' columns indicate from left to right the number of adrenals in which the sudanophobe zone is (*a*) merged with the z. glomerulosa, (*b*) indistinguishable from either z. glomerulosa or z. fasciculata, (*c*) merged with the z. fasciculata.

The blood vessels of the cortex

Since many erythrocytes stain blue with acid haematein (but after pyridine extraction as well, so that this result cannot be taken to indicate phospholipines), the course of the blood vessels in the cortex is often very easy to follow. In addition, in many acid haematein sections, extremely thin-walled blue-stained tubes may be seen (Pl. 2, fig. 5). No nuclei have been observed in their walls, nor have erythrocytes been seen in them. Occasionally they appear after Aoyama's technique.

The general distribution of the blood vessels follows very closely that described by Bennett & Kilham (1940) in the cat, and Gersh & Grollman (1941) in the rat and mouse. The sudanophobe region is less vascular than the z. glomerulosa proper. Occasionally, when, as previously reported (Harrison & Cain, 1947), the fasciculations of the z. fasciculata stretch right to the capsule, the sudanophobe zone can be seen to be supplied partly from the vessels of the z. glomerulosa, and partly from the commencement of the radial vessels of the z. fasciculata.

The thin-walled tubes described above correspond very closely to the 'arteriae medullae', first adequately described by Flint (1900) in the dog, and easily shown by micro-arteriography after the injection of a radiopaque medium (Pl. 2, fig. 6). They appear to lie loosely in spaces presumably filled with tissue fluid. In some

sections they can be seen, often in bundles, passing through the capsule, z. glomerulosa (widely interrupted at this point) and z. fasciculata, either directly into the medulla, or into it after undergoing some convolutions in the z. reticularis. In the medulla they appear to be quite separate from the large blood vessels so abundant there.

DISCUSSION

Histochemistry of the variations

(*a*) *Critique of methods used*

(i) *Sudan Black and Sudan IV.* Lison (1936), who introduced Sudan Black into histochemistry, gives a general discussion of lipoid colorants. These substances are not dyes and cannot *stain*. They colour lipoids simply by dissolving in them; Sudan Black, being the most intensely coloured, is therefore the most sensitive and satisfactory. According to Lison their specificity is perfect. It should be noted that they do not colour pure carotenoids (Lison, 1936, p. 245) which, nevertheless, are lipoids. Cain has shown (1947*a*) that the general pink coloration produced in any section by Sudan IV has no significance, and that Sudan Black is much more satisfactory since faint colours with it are nearly always highly significant. Cain (1947*b*) also points out that *solid* lipoids are not coloured by Sudan Black and similar colorants. This point is of considerable importance. Apart from one incidental remark by Lison (1933, p. 252) the physical condition of lipoids *vis-à-vis* their reactions has been wellnigh ignored in the literature. But it can easily be shown that solid tristearin, or frozen triolein, for example, are uncoloured by Sudan Black, but become intensely coloured on melting. This explains why carotenoids (with melting points in the neighbourhood of 170° C.) do not colour. Similarly, crystals of cholesterol or cholesteryl esters in adrenal sections treated with Sudan Black remain uncoloured. An exception to this rule is cholesteryl oleate, which, being a grease at room temperature, colours intensely as does lecithin, which is also a grease. If a suitable section containing droplets of lipoid (e.g. a transverse section of the leech *Glossiphonia*, Cain, 1947*c*) is chilled to 1° C. and treated with Sudan Black or other colorant (Nile Red is very suitable) at the same temperature, the lipoid droplets are not coloured. On warming, they become coloured intensely. Consequently, although everything coloured by Sudan Black in a section is, or contains, lipoid, it is not certain that everything which is not coloured is not, or contains no lipoid. In the case of the adrenals it appears that the principal uncoloured lipoids are cholesteryl crystals, which in the rat are comparatively few and easily seen by other methods.

(ii) *Nile Blue.* The specificity of Nile Blue has recently been re-investigated (Lison, 1935*a*,*b*; Cain, 1947*b*, 1948*c*). It is not, as formerly thought, and recently restated (e.g. Menzies, 1949), a reagent for unsaturated triglycerides. This conclusion was based on ignorance of the fact mentioned above that lipoid colorants will not colour solid lipoids. The red substance, which Lison (1935*a*,*b*) called 'Nile Red', is a lipoid colorant of the same action as the Sudans. The blue substance (Nile Blue sulphate) is a basic dye. The result of using the two together on tissues is to effect a distinction between 'neutral' and 'acidic' lipoids (Cain, 1947*b*). 'Acidic' lipoids comprise free fatty acids, phospholipines, galactolipines, and chromolipoids, and stain blue with the basic dye Nile Blue sulphate. 'Neutral' lipoids comprise hydro-

carbons, higher alcohols, esters and waxes, all of which are unstained by Nile Blue sulphate but, unless solid, dissolve Nile Red and are coloured by it. Nile Blue sulphate, being merely a basic dye will, as Lison points out, stain everything in a section. One must consider, therefore, only bodies known to consist of nothing but lipoids (usually granules and droplets, although strictly speaking, even in them it is almost impossible to *prove* the absence of other substances). If such a body is coloured pink by Nile Blue (the ordinary commercial dye, which is a mixture of the oxazine sulphate and the oxazone its derivative), then it consists only of neutral lipoids. As to which neutral lipoids it contains, and in what proportions, nothing whatever can be said. If it stains blue, then it contains at least some acidic lipoids, and since the pink colour is very easily masked by the blue, it may contain neutral lipoids as well; at least, their total absence cannot be affirmed. If it is coloured some intermediate shade of purple, then obviously it contains both acidic and neutral lipoids. One might also add that if it takes up Nile Red at all, it must be either a grease or a liquid and therefore is, to some extent, unsaturated (since most naturally occurring lipoids are solid at ordinary temperatures if saturated, and tend to have lower and lower melting-points with increasing degrees of unsaturation). The converse, however, is not true. Because a body is solid and contains only lipoid, it is not on that account composed of saturated lipoids only. The obvious example is cholesterol (m.p. 148·5° C., anhydrous). Since unsaturated triglycerides are common in nature, and since triolein (an example of the class) was widely used by earlier experimenters as a solvent for other lipoids, it came to be thought that only unsaturated triglycerides were coloured pink by Nile Blue, and therefore a pink colour with Nile Blue is specific for unsaturated triglycerides. This view is incorrect.

(iii) *Acid haematein.* Baker's acid haematein test for phospholipines (1946, 1947) has recently been investigated (Cain, 1947*c*), and is considered to be specific for phospholipines provided that Baker's directions are scrupulously adhered to. A blue to blue-black colour seen after the acid haematein test but not after the pyridine extraction control test signifies phospholipine.

(iv) *The modified plasmal test.* A discussion of this method has been given by Cain (1949). For convenience, the technique is repeated here.

(1) Very small pieces of fresh tissue are dropped into a mixture of equal parts of (i) Schiff's reagent diluted with its own volume of sulphur dioxide water; and (ii) a saturated aqueous solution of mercuric chloride contained in a tightly stoppered vessel. Control pieces are placed in the diluted Schiff's reagent only, no mercuric chloride being added. Both lots of pieces are left in the solution for 15 min.

(2) The pieces are removed, rinsed in sulphur dioxide water (to remove excess Schiff's reagent), and dropped into formalin (40 % formaldehyde) in which they are left for 2 hr. All the pieces rapidly become dark violet through the formation of the formaldehyde-Schiff compound.

(3) The pieces are washed in running water for 2 hr. or more until the control pieces are only a very pale lilac. This removes the excess formaldehyde and the formaldehyde-Schiff compound.

(4) Preferably, frozen sections are cut without embedding. If this is not possible, the pieces are embedded in gelatine overnight and the blocks are hardened in formalin. These operations should be carried out quickly.

Only very small pieces of tissue can be used, as penetration by Schiff's reagent is poor.

Results of experiments with rat adrenals. One adrenal from each of eleven rats (immature males and females) was examined by the Feulgen-Verne technique as described by Lison (1936, p. 216), the other adrenal being treated by Baker's acid haematein method. By the latter technique the stage of each adrenal was determined. Most were at stage II, but there was one at IX, one at IV–V and one at I–II. To investigate the progress of the reaction the following procedure was used. Four groups of frozen sections freshly cut, were taken. One was treated immediately with Schiff, another after the prescribed time in mercuric chloride solution. The others were left in water overnight and then similarly treated.

Both groups of sections treated immediately after cutting showed a rather general, very feeble, coloration throughout the cortex, slightly intensified as a rule in the z. glomerulosa and in the outer z. fasciculata just where the heaviest loading of phospholipine was shown by the acid haematein test. The medulla was also feebly coloured. (The significance of this coloration in the medulla is discussed by Cain, 1949.) There was perhaps in one or two sections an extremely slight intensification of the colouring in the sections treated with mercuric chloride, but it was not at all obvious.

Both groups of sections treated after standing overnight in water showed a far more intense coloration, not greater in the sections treated with mercuric chloride. The picture obtained was exactly that seen in the corresponding acid haematein sections, but with the z. glomerulosa even more intense—in other words, it corresponded to the distribution of lipoid droplets rather than, or in addition to, the distribution of phospholipine. The cells are not well fixed by this technique, and the lipoid droplets ran together. Close examination suggested that the lipoid droplets were positive as well as the rest of the cytoplasm. The stain is too feeble and transparent to allow any conclusions as to whether the cytoplasmic stain is due to the mitochondria or the ground cytoplasm or both.

These results confirm the conclusion, already reached, that this technique shows principally (in this case almost entirely) a *pseudoplasmal* reaction, due (Cain, 1949) to autoxidation of various lipoids, not a true plasmal reaction due to the catalysed release of aldehydes from acetalphosphatides.

With the modified plasmal technique given above, the results, as already described, were quite different. The controls were consistently negative (except for a very faint staining in some medullae). The sections treated with mercuric chloride showed a general coloration in the outer parts of the cortex, in which the lipoid droplets appeared not to participate. The fat droplets of attached adipose tissue were also uncoloured, although the cytoplasm of the fat cells was pink. The picture obtained did not vary obviously with the stage of the adrenal. Again, it is not possible to say what components of the cytoplasm and its organelles were responsible for the positive results. (A minor convenience of this method was that mercuric precipitates were formed, presumably by reaction with adrenaline, at the edge of the medulla, outlining it very clearly.)

(v) *Phenylhydrazine techniques.* Although there are many biochemical colour tests for various cortical and other steroids, none are satisfactory in histochemistry.

Several require a preliminary selective extraction and purification to give them their specificity. Others are interfered with by cholesterol or other substances. The only histochemical method is that of Bennett (1939, 1940). This employs phenylhydrazine as a reagent for the $>C=O$ group, and results are considered positive if they can be prevented by extraction with lipoid solvents or treatment with semicarbazide, and if they are paralleled by those obtained with Reichstein's silver reagent. In other words, those regions of the adrenal cortex (or other organ) in which a colour is produced by phenylhydrazine but not after the treatment just mentioned, and in which silver is precipitated, must contain lipoid aldehydes and ketones, which will combine with phenylhydrazine and semicarbazide, reduce silver solution, and be removed by lipoid solvents. A preliminary oxidation was considered necessary to remove ascorbic acid which would otherwise reduce the silver solution. Bennett remarked (1940) that as no other aldehydes with lipoid solubilities had been shown to exist in the cortex, the substances responsible must be ketosteroids. (The test is sometimes said to be for 17-ketosteroids; Reichstein & Shoppee (1943) mention in a very long list of cortical steroids only four (p. 368) that are 17-ketosteroids, and three of these are possibly oxidation products which appear during the process of extraction. The fourth is oestrone. Many other ketosteroids are listed, principally with one of the ketonic groups at position 20, the rest being on other rings.)

The depth of colouring in Bennett's test was improved by Pechet (1946; see Yoffey & Baxter, 1947), who used 2, 4-dinitrophenylhydrazine which is much more intensely coloured. Camber (1949) has suggested diazotization of the resulting hydrazones to intensify the colours produced still more.

Pollock (1942) found that Bennett's reaction was intense in the interstitial cells of the testis. Aboim (1943) noticed the remarkable similarity between results obtained with the 'plasmal reaction' (actually the *pseudoplasmal* reaction) and with Bennett's reaction in the adrenal cortex of many animals. Albert & Leblond (1946) found in the course of an extended study of many tissues, that the two methods always gave identical results, and worse, that the mercuric chloride used for intensifying the plasmal reaction could be used to give a similar intensification of Bennett's reaction. Gomori (1942) had pointed out that while Bennett's method could show ketosteroids, it would also show plasmal aldehydes, which were lipoidal aldehydes and would behave in the same way as ketosteroids; the preliminary oxidation with oxygen or iodine would cause the plasmal aldehydes to be liberated exactly as mercuric chloride does in the plasmal reaction. (Mercuric chloride is a mild oxidizer, but the release of plasmal is a hydrolysis, not an oxidation; oxidation would convert the very unstable plasmal aldehydes to the corresponding—and unreactive—fatty acids.) Albert & Leblond considered that their results confirmed Gomori's suggestions.

These criticisms of Bennett's technique were not accepted by Dempsey & Wislocki (1944, 1946), who maintained that the plasmal reaction could itself be given by certain, unspecified, ketosteroids. This unlikely possibility is not supported by the work of Boscott, Mandl, Danielli & Shoppee (1948). Dempsey & Wislocki added birefringence and ultra-violet fluorescence to the tests used by Bennett, and considered that all these tests used together were sufficient to demonstrate the presence of ketosteroids.

Claesson & Hillarp (1947) investigated experimentally the evidence that oestrogens

could be identified histochemically in the ovary. In an excellent paper, they demonstrated the invalidity of the assumption by Dempsey & Wislocki that all the positive results obtained in a particular tissue, or even a particular granule, must be caused by the same substance. They showed that none of the tests, taken singly or in combination, could show the presence of ketosteroids in the tissues they examined. Claesson & Hillarp noticed that the phenylhydrazine reaction became positive only after a fixation of 24 hr. or more, unless iodine were used as a mild oxidizer; this immediately suggests a comparison with the Feulgen-Verne pseudoplasmal reaction which, as already shown, is caused by oxidation of unsaturated, principally aliphatic, lipoids; they found that their results were not affected by short treatments with mercuric chloride. It seems most likely that the intensification observed by Albert & Leblond was, in fact, due to the liberation of some plasmal from acetalphosphatides still left in the tissues.

Rogers & Williams (1947) mention, in the course of observations on human adrenals, that with phenylhydrazine 'the fat contained in signet ring cells outside the capsule is also yellow'. With 2, 4-dinitrophenylhydrazine the colouring of fat external to the capsule is even more marked in rat and mouse adrenals (Cain, unpublished observation), the colour of this derivative being much stronger than that of phenylhydrazine. Ordinary fat droplets can be coloured by it without any preliminary oxidation. Presumably phenylhydrazine will colour them also but very much more weakly.

Accordingly, some adrenals (adult male rat) were investigated by means of Pechet's modification as given by Yoffey & Baxter (1947). The picture obtained corresponded exactly to that given by the pseudoplasmal reaction. In addition, test-tube experiments showed that 2, 4-dinitrophenylhydrazine and its hydrochloride, and the coloured substances resulting from their spontaneous oxidation (with atmospheric oxygen) will dissolve, sometimes rather slowly, in such liquid lipoids as triolein, ethyl oleate, and oleic acid. It is not denied that phenylhydrazine and its derivatives can and do form hydrazones with suitable carbonyl groups in the tissues, but the mere production of a colour in a lipoid droplet or in cytoplasm is no evidence that a hydrazone has been formed. It may have been produced by simple solution. Further, all derivatives of hydrazine are *ipso facto* extremely strong reducing agents and can prevent atmospheric oxidation of fats. They may react with active —C=C— bonds as well as active >C=O bonds, and with all sorts of oxidizers. Now, as has been shown above, many unsaturated lipoids undergo autoxidation if exposed to air, to give rise to very various compounds some of which (e.g. the hydroperoxides) are themselves oxidizing agents. It is quite likely, then, that the action of *mild* oxidizers on ordinary fat droplets should cause an intensification of the colours produced with hydrazine derivatives; but that again is no evidence that keto groups are involved. Our results support the belief that the phenylhydrazine reaction and its modifications may be caused in part by ketosteroids, but there is no means of distinguishing this part of the results from those caused by various other substances, and by simple solution. Some of the supplementary tests brought forward by Danielli (1949) to support his aldehyde reactions are open to such objections as have just been made to the supplementary reactions for Bennett's test.

(*b*) *Interpretation of histochemical results*

The results described above allow certain histochemical conclusions to be stated, the considerations just outlined being kept in mind.

The total lipoid content of the adrenal varies considerably, being a maximum at stages IV–V and a minimum at stage IX. This variation is due almost entirely to the changes in the z. fasciculata, the changes in the z. glomerulosa being much smaller. The principal variation in the z. fasciculata is due to the appearance and disappearance of the lipoid droplets. The amount of *histochemically* detectable phospholipine also varies in correlation with the amount of lipoid droplets.

The lipoid droplets of the cortex frequently colour pink with Nile Blue, and then must be considered to contain only neutral lipoids (higher hydrocarbons, alcohols and esters), but it is impossible to say which. Since they colour at all at room temperature, they must be liquid or greasy. A very little experimenting shows that they are quite liquid. It is reasonable to suppose, therefore, that they contain a certain amount of unsaturated lipoids of low melting-point, holding others in solution. As previously reported (Harrison & Cain, 1947), all the droplets are positive to Liebermann's reaction, which is specific for cholesterol and its esters (and a very few closely related sterols). These, except cholesterol oleate, are crystalline at room temperature and therefore uncoloured by Nile Blue or the Sudans. (It is, of course, an easy guess that the liquid solvent lipoids are triglycerides containing some proportion of unsaturated acid radicles, since these are so common in fat.)

When the droplets are uncoloured, no conclusion can be drawn, except that they are solid. When they are coloured blue or some intermediate purple by Nile Blue, then acidic lipoids must be present as well. Galactolipines have never been reported in any quantity from the cortex, nor have free fatty acids. It is probable, therefore, that phospholipines (perhaps including acetalphosphatides) are responsible. This is confirmed by the rather weak positive results obtained with acid haematein.

Acid haematein shows the principal demonstrable phospholipine to be in the mitochondria of the cells containing, or about to contain, large numbers of lipoid droplets. The rest is found in the discharge bodies, the externa of the spheroids, and (abundantly) the cytoplasm of the dead and dying cells in the z. reticularis.

The modified plasmal technique shows that acetalphosphatides are rather uniformly distributed in the middle and outer z. fasciculata, the sudanophobe zone and the z. glomerulosa. They are not present in the lipoid droplets (and nuclei). Their exact location in the cell is uncertain. Their distribution does not vary greatly in the different stages, according to our observations.

Nothing can be said about the distribution of any sort of steroid or sterol, except cholesterol (and its esters). The cholesteryl radicle is abundant in all the lipoid droplets; occasionally it is found in crystals.

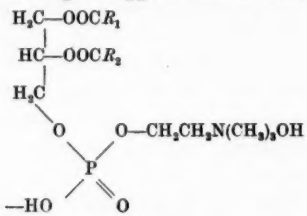
Interpretation of the cytological appearances

Several of the cytoplasmic bodies, especially the spheroid complexes described, bear a close resemblance to appearances found by Hoerr (1936*a*, *b*) in the guinea-pig, by means of osmium tetroxide, which he regarded as artefacts. However, the techniques used above included fixation and give results, particularly as regards the

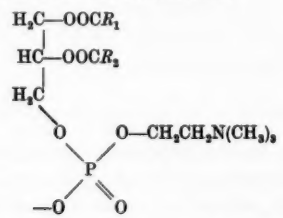
lipoid droplets, which Hoerr regards as genuine and characteristic of good fixation. In order to compare our results with his, some of the techniques described were carried out on guinea-pig adrenals, and gave good results. Again, the use of Sudan Black confirmed the presence of spheroid complexes in rat adrenals, and this method of colouring has none of the disadvantages of impregnation with osmium tetroxide. A particularly interesting feature of the guinea-pig adrenal is that in the z. reticularis the nucleus of most cells was capped by a dense mass of bodies, heavily positive with acid haematein, but apparently with an unstained internum. These resemble in disposition the Golgi apparatus, as seen (in a few cells only) in Aoyama preparations, so closely that their identity cannot be doubted. This phenomenon is seen only very rarely in the rat, and there are many other points of difference as well. But it is possible to demonstrate in a few cells of the middle z. fasciculata a certain number of spheroid complexes in the guinea-pig. The result of comparing the guinea-pig and rat adrenals is to confirm that the various appearances seen with these techniques occur under conditions of very good fixation, and have no relation to the artefacts produced with osmium tetroxide.

The cytological interpretation of the variations may be tentatively interpreted as follows. Cells arising in the sudanophobe zone pass outwards to form the z. glomerulosa or inwards to form the z. fasciculata (and z. reticularis). The transition from young cells to adult members of the zones is gradual for the z. glomerulosa, rapid for the z. fasciculata. As the cells pass inwards to the z. reticularis they undergo one (or more) series of changes.

(1) The mitochondria become intensely positive to acid haematein. This appears to mean that the phospholipine contained in the mitochondria is in some form of combination, presumably with their pentose-nucleic acid, which prevents it from forming a compound with chromic oxide. In this special state, however, the combination is partly or wholly broken down, and the phospholipine can now be mordanted and stained. One might suggest that in the case of lecithin:



which behaves in the free state as the zwitterion:



the combination with nucleoprotein involves at least the O—, so that the chromium

cannot form a chelate compound as is general with mordants (see, for example, Sudborough, 1941, p. 708).

Mitochondria of many cells are positive to acid haematein (Baker, 1946; Cain, 1947*a*, p. 157, 1947*c*, p. 468), especially those of certain gland cells, of absorptive cells (for example in the intestine) and of excretory cells (nephridial cells in leeches, convoluted tubules in the mouse). In fat cells, in which the mitochondria are supposed to be extremely numerous and minute (Guyon, 1924), the whole cytoplasm is positive. In view of the known importance of phospholipines as intermediary metabolites, histochemically demonstrable (and therefore probably specially reactive), phospholipines might be considered as indicating some special metabolic activity.

That other mitochondria are devoid of phospholipine is extremely unlikely, and since large quantities of it are visible in dying cells in the cortex, some of which appears to come from mitochondria, it is reasonable to conclude that after the secretory phase has passed, the phospholipines of the mitochondria return to a state of more intimate combination.

(2) Very soon after the mitochondria become positive to acid haematein, large numbers of very regular lipoid droplets, usually consisting of neutral lipoids only, and invariably containing the cholesteryl radicle in large quantities, appear in the cell. In this state the cell very much resembles one of adipose tissue in the rat, and in the leech *Glossiphonia*, with the differences that there is one fat globule in the fat cell instead of many, and that in the fat cell the phospholipine cannot be seen for certain to be confined to the mitochondria (which are much smaller than those of the adrenal cortex cells). The constancy of pattern of fat cells in such unrelated animals as a rodent and a leech suggests that one sees in it no species-specific elaborations, but merely the minimum necessary arrangement for storing fat. It is therefore reasonable to suggest that the adrenal cortex cell in this state is also specially arranged for accumulating fat, and that the presence of large quantities of histochemically demonstrable phospholipine is necessary for this purpose. Our results entirely confirm those of Hoerr (1936*b*) that the mitochondria do not become or give off fat globules, and that intermediate appearances suggesting such a process are due to bad fixation.

The Golgi apparatus does not appear to hypertrophy in the z. fasciculata during the secretory phase, and gives no indication of playing any special part in it.

(3) The lipoid droplets disappear from the cell nearly completely, and more or less simultaneously the mitochondria become negative to acid haematein. It seems extremely likely that the droplets which are known to contain cholesterol in solution may be the carriers for the cortical steroids; probably they are broken up into minute droplets, of the order of size of chylomicrons, and emitted from the cell. There is no evidence of large droplets leaving the cell as such.

(4) The cell, now in the discharged state, contains a number of remarkable bodies, the discharge bodies, containing histochemically demonstrable phospholipine. These appear to become more and more positive to acid haematein with the passage of time. It is possible that this slow process may represent a breaking-down of the same nature as the rapid degenerative process (with release of phospholipines) at cell death. The relation of the discharge bodies to the mitochondria and Golgi

apparatus is uncertain; they appear to be related to the mitochondria if to anything else in the cell. It is just possible that the composite bodies may represent effete mitochondria in the course of breaking down; perhaps the dark granule represents a mitochondrion, and the attached body an effusion similar to the 'archoplasm', well known in molluscan nerve cells (Brambell & Gatenby, 1923; Thomas, 1947).

The spheroid complexes, which may also occur in cells in the secretory state, agree extremely well with single elements of the Golgi apparatus as described by Baker (1944) and later workers (Cain, 1947*a*, 1948*b*; Foster, 1947; Thomas, 1948). The smallest are seen in cells of the outer z. fasciculata, the largest in the z. reticularis, in which they were very common in nearly all the adult rats examined. If their usual increase in size from the outer to the inner cortex indicates individual growth, as seems extremely likely considering that they also increase in numbers per cell, then the internum must be regarded as a growing granule of secretion. These interna are neither birefringent nor fluorescent with ultra-violet light. If they were lipoid and solid they would almost certainly show some birefringence. They do not colour with Sudan Black. Consequently, it is very likely that they are not lipoids. The spheroid bodies are not visible in dying cells; they may be discharged from the cell before death.

If the spheroid complexes belong to the Golgi apparatus, why are they not demonstrated by Aoyama's technique? According to Baker (1944) the affinity of the Golgi apparatus for osmium tetroxide and silver nitrate is largely due to its content of phospholipine (excluding sphingomyelin), of which there is also a high concentration in the outer zones of these complexes. It may be that on occasion mitochondria are more readily impregnated with silver and osmium than the Golgi apparatus (Thomas, 1947; Cain, 1948*b*), so that failure of the latter to impregnate does not prove definitely that it is *not* the Golgi apparatus. Further, Aoyama's fixative is bad for the adrenal cortex; it may destroy the spheroid complexes, as it does the regularity of the lipoid droplets.

In addition the outer zones or externa of the spheroid complexes contain other lipoids than phospholipines, for they stain very strongly both with Sudan Black and with acid haematein, whereas it can be shown that the enormous mitochondria in nephridial cells of *Glossiphonia*, containing large quantities of phospholipine, react strongly with acid haematein but are coloured only medium grey by Sudan Black. Since silver methods will not impregnate fat droplets to any great extent it is possible that phospholipines dissolved in other lipoids of the outer zone become inaccessible to the impregnating solution.

The discovery, mentioned above, of cells in the z. reticularis of the guinea-pig in which the Golgi apparatus is extremely positive with acid haematein, and appears to be made up of numerous spheroids with positive externa and negative interna, goes far to confirm the views just put forward, namely that the spheroid complexes belong to the Golgi apparatus.

The spheroid complexes are not recognizable in dead cells of the z. reticularis (in the rat) and occasionally may be absent from some others as well. This, coupled with their general increase in size towards the z. reticularis, suggests that when fully formed they may break down, probably emitting the interna as granules of a specific secretion as do the components of the Golgi apparatus in many glandular tissues

(Hirsch, 1939). If this is so, then the wide z. reticularis must be regarded as a specially secreting zone, as well as the z. fasciculata and z. glomerulosa. This might explain its surprising width in adult rats. In juveniles the z. fasciculata is renewed by proliferation at least as rapidly as in the adult, yet its inner border often shows no reticulation at all. Dead cells may often be found there but apparently they disintegrate rapidly; there is no senescent period, and they do not accumulate to form a well-defined z. reticularis before dying. It is rather remarkable that in the adult there should be such a wide z. reticularis, especially as the dying cells are still as a rule next the medulla, and do not extend throughout the zone; it is not, so to speak, a mortuary specially enlarged to meet the needs of the increased cell-population. But if it is a special secretory zone, then its development is explained.

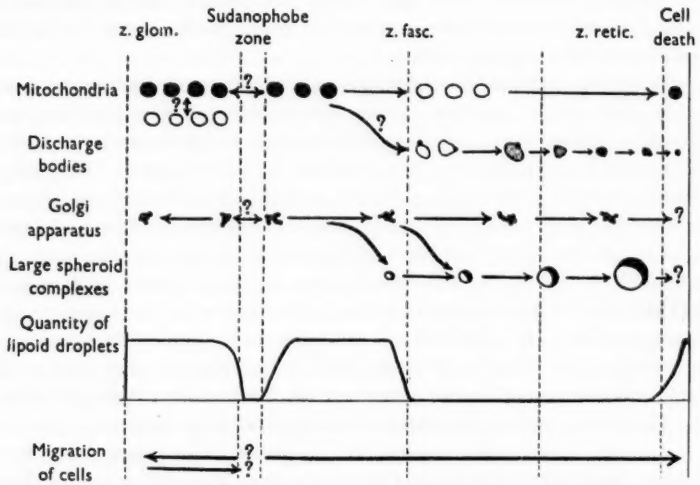
The relationship of the caps and chains mentioned above remains rather problematical as these bodies were so seldom seen. Both appear to be irregular manifestations of the Golgi apparatus.

The z. glomerulosa appears to undergo a similar but much less complex series of changes. In cells of the secretory phase the mitochondria are heavily positive. Lipoid droplets appear very early, and, in contrast to those of the z. fasciculata, do not disappear immediately the mitochondria become negative. They appear to be discharged gradually over a long period, and this zone is very rarely devoid of droplets. Few discharge bodies are seen (none composite) and no spheroid complexes. The view so far adopted here that the z. glomerulosa is proliferated from the sudanophobe zone and moves outwards is based on the work of Baxter (1946). However, Mitchell (1948) denies the occurrence of any cell division in the sudanophobe zone in immature animals. He also considers that the contention of Zwemer and others that the cortex arises from 'indifferent cells' in the capsule may be true for the embryo but certainly is not for the immature rat from about a fortnight after birth onwards. He states that in immature rats treated with colchicine the arrested mitoses are found only in two regions, the z. glomerulosa proper, and the outer z. fasciculata; moreover, the mitoses of the two regions have different and distinctive features. The sudanophobe zone he believes to be merely a zone of compression. He mentions also that the mitotic rate, never very high, falls off rapidly with age. It is unlikely, therefore, that if cyclical changes occur in the adrenal cortex, each complete cycle of secretion corresponds to the complete renewal of the z. fasciculata.

In support of theories envisaging a single series of cells progressing from the capsule or nearby to the edge of the medulla is the observation that although dead cells are frequent in the innermost z. reticularis, and may be found scattered through the z. fasciculata, they are rarely, if ever, seen in the z. glomerulosa, certainly never in the sudanophobe zone. These observations confirm those of Williams (1945), made on isolated adrenal cortical cells of the rabbit in autogenous grafts. But it is not certain that the cells of the z. glomerulosa require replacement as often as those of the z. fasciculata; the mitoses of the z. glomerulosa which, as Mitchell remarks, decrease in number with age, may be sufficient merely to maintain the continuity of the glomerulosal shell over the z. fasciculata as the gland grows, in which case the sudanophobe zone might well be one of compression. In favour of this view is the observation that in several adrenals the sudanophobe cells contained only lipoid droplets whereas those of the z. glomerulosa contained both droplets and

mitochondria. The sudanophobe cells might be at a later stage in that case than those of the z. glomerulosa. Similarly, the apparent disappearance of the sudanophobe zone, recorded above, because its cells were as heavily laden with lipoid droplets as those of the z. glomerulosa, would not suggest that it is a 'juvenile' region.

The cytological evidence presented above appears to confirm the *functionally* separate nature of the z. glomerulosa, which is based on the work of Deane & Greep (1946) and others. One might, therefore, expect an independent recruitment for this zone, and a complete separation from the z. fasciculata. With the necessary reservation that the question is still open, this is the standpoint adopted by us. On this hypothesis, the various cytological phenomena and processes of the cycle can be represented as in Text-fig. 6.



Text-fig. 6. Diagram of the relations between various cytoplasmic bodies. Mitochondria are shown as black or white according as they are positive or negative respectively to acid haematein. The mitochondria in the z. fasciculata can give rise to certain discharge bodies after the lipoid droplets have disappeared from the cells. At the same time the Golgi apparatus can give off large spheroid complexes with lipoid externa. In the z. glomerulosa these processes do not seem to occur to any great extent.

Correlation of the histological appearance with physiological investigations

(*a*) *Correlation of function with zone morphology*

From the work of Deane & Greep (1946) it appears that the z. glomerulosa is markedly different from the z. fasciculata in function as well as structure. The salt-regulating fractions of adrenal cortical secretion (e.g. desoxycorticosterone) are probably formed in the z. glomerulosa, while the carbohydrate-regulating principles (i.e. the 11-keto- or hydroxy-corticosterones) are secreted by the z. fasciculata. As a generalization this theory of adrenal cortical function is obviously of great importance. In a gland as complex as the adrenal cortex, however, with its many different secretions, there may be certain objections to an absolute demarcation of function in this manner. Thus both corticosterone and dehydrocorticosterone have

been shown to produce (temporary) sodium and chloride retention (Thorn, Engel & Eisenberg, 1938), although desoxycorticosterone seems to have no effect on carbohydrate metabolism, while the pituitary adrenocorticotrophic hormone, although apparently not causing salt retention in the rat (producing mainly an increased secretion of 11-keto- or hydroxy-corticosterones), does so in man (Bergner & Deane, 1948). Our results do, nevertheless, confirm that the z. glomerulosa and z. fasciculata are cytologically and histochemically very distinct zones. The evidence of the more regular and continuous discharge of lipoid droplets which we have found in the z. glomerulosa would correspond closely to the life-maintaining, salt-regulating function of this zone. The z. fasciculata, from its histochemical appearance, may become almost completely depleted of phospholipine (stage IX); this has never been found to be the case in the z. glomerulosa.

The z. reticularis has been shown to undergo pronounced changes during the breeding season or pregnancy in certain animals (Bourne, 1934*b*; Zalesky, 1934; and others). According to Bourne & Zuckerman (1941), however, the changes in the adrenal cortex during normal or artificial threshold oestrous cycles in the rat are mainly evident in the z. fasciculata; changes in the z. glomerulosa were slight and not significant. If Deane & Greep's (1946) theory be correct, it is therefore less likely that the variations in response of the reproductive tract in the rat are due to cyclical hydration and dehydration by salt-regulating compounds of the adrenal cortex (Long & Zuckerman, 1937) than that these changes are brought about by cyclical variations in the secretion of oestrogenic, androgenic or progestational hormones.

(*b*) *Time relations of the variations in the adrenal cortex*

In continuing our investigation of the histochemical variations in the rat adrenal cortex as seen by acid haematein it was thought advisable to examine large numbers of rats by the method already adopted, namely by killing litter-mate rats from day to day. This method was continued since the variation in histochemical appearance of the adrenal cortex in rats killed in this way was greater than that in rats from a litter killed simultaneously. The findings in the litters of rats killed simultaneously are detailed in Table 3, while the stage numbers of the rats from litters whose members were killed from day to day are shown in Table 4.* It can be seen that the variation in stage numbers in such rats killed *seriatim* is much greater, in the majority of litters, than that in a litter of rats killed simultaneously. In view of the indications in Table 3 that at a given moment siblings of like sex are at the same or very nearly the same stage, this variation suggested that there might be a cycle of secretion such that siblings are in the same phase.

There is ample evidence for cyclical changes occurring in the adrenal cortex. Fraser (1929) has demonstrated a cycle of change in the interrenals of an Elasmobranch. In the rat, Bourne & Zuckerman (1941) have clearly demonstrated changes in the adrenal cortex in a period corresponding to the oestrous cycle, while in man Pincus, Romanoff & Carlo (1948) have produced evidence for a diurnal rhythm in the excretion of 'neutral reducing lipids' and 17-ketosteroids. Any cycle of secretion in

* Owing to the different classification in this paper, the stages in these tables do not correspond to those in our previous communication. The results described in the previous paper were reclassified for use in this.

the adrenal cortex would therefore most likely be diurnal or of the order of 4–6 days. When the data in Table 4, which displays the results for rats killed *seriatim*, are examined, it is obvious that there is no evidence for a cycle of 4–6 day periodicity; the variation in stages, although much greater in rats killed *seriatim* than simultaneously, is nevertheless not as large in some litters (e.g. litter N). In order to exclude a diurnal rhythm, the rats in some litters were therefore killed 2- and 4-hourly (Table 5). These data also give no evidence for a diurnal rhythm in adrenal cortical secretion. There is also no definite evidence that members of one sex in a given litter are at the same stage as those of the opposite sex (see litters F and VIII).

Table 3. *Analysis of immature rats killed simultaneously*

(Each rat is represented by its stage number.)

Litter	Sex	Body weight (g.)	Stage number of rats in litter
A	♀	26–31	I–II; I–II; early II; early II
B	♀	19–23	Six rats, all stage I
X	♀	22–30	II–III; IV; III–IV; III
Y	♀	20–22	II–III; II; II
Z	♀	27–30	III; III; II
F	——	21–27	II; II; II; II (♀). I; I; IX–I (♂)

Since it was not possible to obtain definite evidence for a cycle of secretion by this procedure, it was therefore considered that these variations in histochemical appearance may be due to variations in the method of killing the animal, the only obvious variable factor in the experimental method. Most of the animals were killed by chloroform, and since this substance could conceivably have an influence on adrenal cortical lipoids, other methods were used. The members of litter VII, for example, were killed by decapitation, and the members of litter IX by coal-gas; as seen from Table 4 the variation in stage number amongst the members of these litters varied as much, if not more, than that in the other litters killed by chloroform overdosage. These two methods still do not rule out the effects of variability of trauma, however, which may become evident as manifestations of the first stage ('alarm reaction') of the general adaptation syndrome (Selye, 1946), although histological changes in the adrenal cortex are stated by Selye (1946, p. 153) to take several hours to develop, and even amputation of limbs is considered only a mild alarming stimulus (p. 131). In three further litters (D, E and F, Tables 3 and 5) the rats were therefore killed by means of a constant dose of nembutal (200 mg./kg.) injected intraperitoneally. By this method it was found that the time between injection of the nembutal and cessation of respiration (when the adrenals were immediately removed) was quite constant for the members of one sex in a given litter. Thus, all the female rats in litter D died in 200 sec., while all the male rats in litter E (except one, taking 170 sec.) died in 160 sec. Yet variation is still evident in litters D and E, while the members of either sex in litter F show uniformity of stage appearance.

Tables 3 and 4 suggested that litter-mates are more closely alike at any one time than at different times. In Table 6, stages I–V (the secretory phase) are opposed to stages VI–IX (the discharge phase). (Intermediate groups are included in the previous groups, e.g. III–IV in III.) This table shows a significant difference in the proportions of the two groups between rats killed simultaneously and rats killed

Table 4. *Analysis of rats killed* seriatim, *and daily or on alternate days at* 10 *a.m.*

Each rat is represented by its stage number and sex. The vertical columns represent days.)

Litter	Body weight (g.)	Stage number and sex of rats in litter												
A1	13–22	VIII ♀	VIII ♀	I–II ♀	VIII ♀	II ♀	III ♂	III–IV ♂	V ♂	III ♂	—	—	—	—
B1	17–23	VIII ♂	VIII–IX ♀	II ♂	VIII–IX ♀	IX ♀	II ♂	III–IV ♂	—	—	—	—	—	—
P	120–146	II ♂	II ♂	III–IV ♂	III–IV ♂	—	—	—	—	—	—	—	—	—
Q	32–42	VIII ♂	II ♂	II ♂	III ♂	III ♂	—	—	—	—	—	—	—	—
R	44–60	II ♀	II ♀	II ♀	VIII ♀	—	—	—	—	—	—	—	—	—
VII	25–36	VII ♀ early	—	VII ♀ early	—	II ♀	—	III–IV ♀	—	II ♀	—	—	—	—
N	41–51	II ♂	—	II ♂	—	II ♂	—	VIII–I ♂	—	II ♂	—	—	—	—
VIII	50–67	II ♀	—	II ♀	—	II ♀	—	I–II ♀	—	IV–V ♂	II ♀	—	—	—
IX	20–27	IV ♀	—	II–IV ♀	—	VIII ♀	—	VIII ♀	—	VII–VIII ♀	—	IX ♀	—	VII–VIII ♀
B2	81–106	I ♀	—	I ♀	—	IX ♀	—	IX–I ♀	—	VII ♀	—	VIII–IX ♀	—	—
W	24–43	II ♀	VI ♀	VI ♀	III–IV ♀	V–VI ♀	II ♀	—	—	—	—	—	—	—

Table 5. *Analysis of immature rats killed* seriatim, *and two- or four-hourly*

(Each rat is represented by its stage number and sex.)

Litter	9 a.m.	11 a.m.	1 p.m.	3 p.m.	5 p.m.	7 p.m.	9 p.m.	11 p.m.	1 a.m.	3 a.m.	5 a.m.	7 a.m.	9 a.m.	11 a.m.	1 p.m.	3 p.m.	Body weight (g.)
H	VIII ♀	VIII ♀	I–II ♀	II ♀									IX ♂	IX ♂	IX ♂	—	21–26
J	II ♂	IX–I ♂	early II ♂	early II ♂									IX–I ♀	IX–I ♀	IX–I ♀	—	23–28
K	I–II ♂	I–II ♂	I–II ♂										II ♀	early II ♀	—	—	25–39
L	I–II ♀	early II ♀	early II ♀	I–II ♂	—	—	—	—	—	—	—	—	—	—	—	—	26–30
D	—	—	—	IX–I ♀	—	early II ♀	—	IX–I ♀	—	I–II ♀	—	I ♂	—	IX–I ♂	—	early II ♂	19–24
E	—	—	—	—	—	IX–I ♂	I–II ♂	I ♂	I ♂	I ♀	III ♀	—	—	—	—	—	32–34

daily or on alternate days, an effect which seems indicative of a real tendency for siblings killed simultaneously to fall into the same groups. If stages VIII to III are opposed to stages IV–VII (the former grouping containing all the highest frequencies, as can be seen from Table 1) as in Table 7, the same tendency is evident, although the difference here is not quite statistically significant.

Table 6. *Distribution of stages according to cytological appearance*

	Stages I–V (including IX–I) (Secretory)	Stages VI–IX (Discharge)	Total
All rats	113	41	154
Rats killed daily or on alternate days	42	22	64
Rats killed simultaneously	27	0	27
Rats killed 2-hourly	21	8	29
Rats killed 4-hourly	7	0	7
Rats killed at random (including operated rats)	16	11	27
Rats killed at random (excluding operated rats)	12	9	21

Table 7. *Distribution of stages according to frequency*

	Stages VIII–III	Stages IV–VII	Total
All rats	127	27	154
Rats killed daily or on alternate days	53	11	64
Rats killed simultaneously	26	1	27
Rats killed 2-hourly	29	0	29
Rats killed 4-hourly	7	0	7
Rats killed at random (including rats having had operative interference)	12	15	27
Rats killed at random (excluding rats having had operative interference)	8	13	21

If the intra-litter similarity be regarded as established, careful inspection of Tables 4 and 5 gives a clear decision against a regular cycle with a period of 4–6 days or of 24 hr.: comparison of siblings does not show the sequence of stages consistent with such a cycle. On cytological evidence it seems unlikely that irregular jumps from one stage to another not necessarily adjacent in the series can occur, yet some fluctuation is strongly indicated by the combined evidence of Tables 3 and 4. The only remaining question is whether the members of a litter go through stages I, II, ..., VIII, IX, I, together in sequence but with an irregular periodicity, or whether they do not always follow the whole sequence but instead may sometimes reverse the direction of change. The statistical evidence is not contrary to either of these possibilities, nor can it, by its very nature, favour either. The cytological evidence definitely points to the former. Of course there is no evidence that different litters are in step with one another, nor is there any obvious reason for expecting them to be.

The evidence given above is severely limited because with these methods of investigation it is possible to make only one observation on each animal, a state of affairs already commented on by Carleton (1934).

It should be noted that in investigating variations in the adrenals after injection, gonadectomy, or hypophysectomy, it must be shown that the appearances attributed to the effects of these procedures are clearly distinguishable, cytologically or statistically, from the normal variations described above. In some investigations animals have been killed at various intervals following experimental procedures, and after comparison with a few controls which are not litter-mates, the differences seen between the adrenals of the treated animals and those of the controls have been attributed directly to experimental interference. Clearly, such changes may well be within the normal variation as shown by our investigation. Thus some of the appearances shown by Deane & Greep (1947, fig. 8) and Bergner & Deane (1948, figs. 17–19) have been seen by us in normal animals. Bergner & Deane's fig. 17 resembles closely the appearance shown by us (Harrison & Cain, 1947, Pl. 1, fig. 5) in a normal rat in dioestrus.

SUMMARY

1. The variations in the lipoids of the adrenal cortex of the albino rat indicate a secretory phase and a discharge phase. In the secretory phase numerous lipoid droplets are present in the cells of the z. fasciculata. The mitochondria present an appearance of special physiological activity in relation to the lipoid droplets of the z. fasciculata, but do not appear to produce them. In the discharge phase the lipoid droplets are very few.

2. The z. glomerulosa almost always contains lipoid droplets, which are therefore probably discharged more evenly and continuously than are those of the z. fasciculata.

3. The so-called sudanophobe zone is always present, but not always sudanophobe. It is distinguishable primarily by the comparatively small amount of cytoplasm in each cell. It may be a growth zone, transition zone, or compression zone.

4. The z. reticularis is represented in the immature animal, but fully developed only in the mature. Its cytology indicates that it is a zone of secretion, not merely of senescence and degeneration.

5. The statistical evidence presented indicates that, in animals of a litter killed simultaneously, the adrenals resemble each other; in rats of a litter killed on successive days, however, the adrenals show marked changes, which have no regular periodicity. The cytological evidence suggests some sort of sequence in the variations, but gives no definite evidence for a cycle of regular periodicity, although the possibility of the occurrence of cyclic changes with an irregular period must be considered.

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EXPLANATION OF PLATES

In all the figures only a strip of the adrenal cortex is shown, the capsule being uppermost in each figure. The adrenals shown in Pl. 1, figs. 1–8, and Pl. 2, fig. 1, are all from immature rats of the same litter (A 1) killed consecutively and daily, and show, in order, the histological picture after the acid haematein method in the individual rats. The magnification in all the figures of Pl. 1, figs. 1–8 and Pl. 2, figs. 1–5, is ×160.

Plate 1

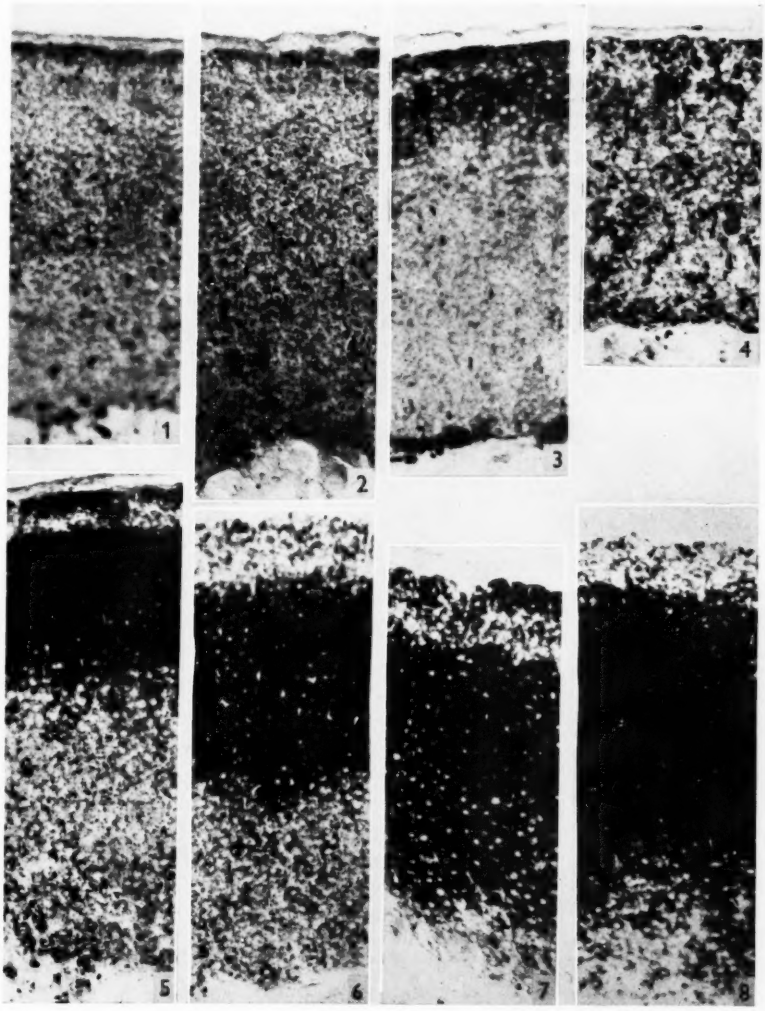
Fig. 1. Female rat, weight 14 g. Stage VIII.
Fig. 2. Female rat, weight 13 g. Stage VIII.
Fig. 3. Female rat, weight 13 g. Stages I–II.
Fig. 4. Female rat, weight 13 g. Stage VIII. Stained blood corpuscles are clearly seen scattered over the section.
Fig. 5. Female rat, weight 18 g. Stage II.
Fig. 6. Male rat, weight 16 g. Stage III.
Fig. 7. Male rat, weight 22 g. Stage IV.
Fig. 8. Male rat, weight 21 g. Stage V.

Plate 2

Fig. 1. Male rat, weight 21 g. Stage III.
Fig. 2. Adrenal cortex of an immature female rat (weight 28 g.), stained by the acid haematein method, showing stage VI.
Fig. 3. Adrenal cortex of immature female rat (weight 25 g.), stained by the acid haematein method, showing stage VII.
Fig. 4. Adrenal cortex of immature male rat (weight 26 g.), stained by the acid haematein method, showing stage IX.
Fig. 5. Adrenal cortex of immature female rat (weight 24 g.), stained by the acid haematein method. A dark-staining linear structure, comparable in position to an arteria medullae, can be seen passing right through the cortex into the medulla in the upper part of the figure. A portion of another is seen in the lower right-hand corner of the figure.
Fig. 6. Micro-arteriograph of a 0·5 mm. section of the adrenal of a cat after the injection of thorotrast into the thoracic aorta. Several blood vessels can be seen around the periphery of the cortex, and two arteriae medullae are clearly seen passing through the cortex, without branching, to reach the medulla. At the juxtamedullary border of the cortex are seen leashes of capillaries collecting into large medullary vessels. (×10.)

OBITUARY NOTE

We have just heard with great regret of the death in Oxford of Professor S. E. Whitnall. A notice *In Memoriam* will appear in a later issue.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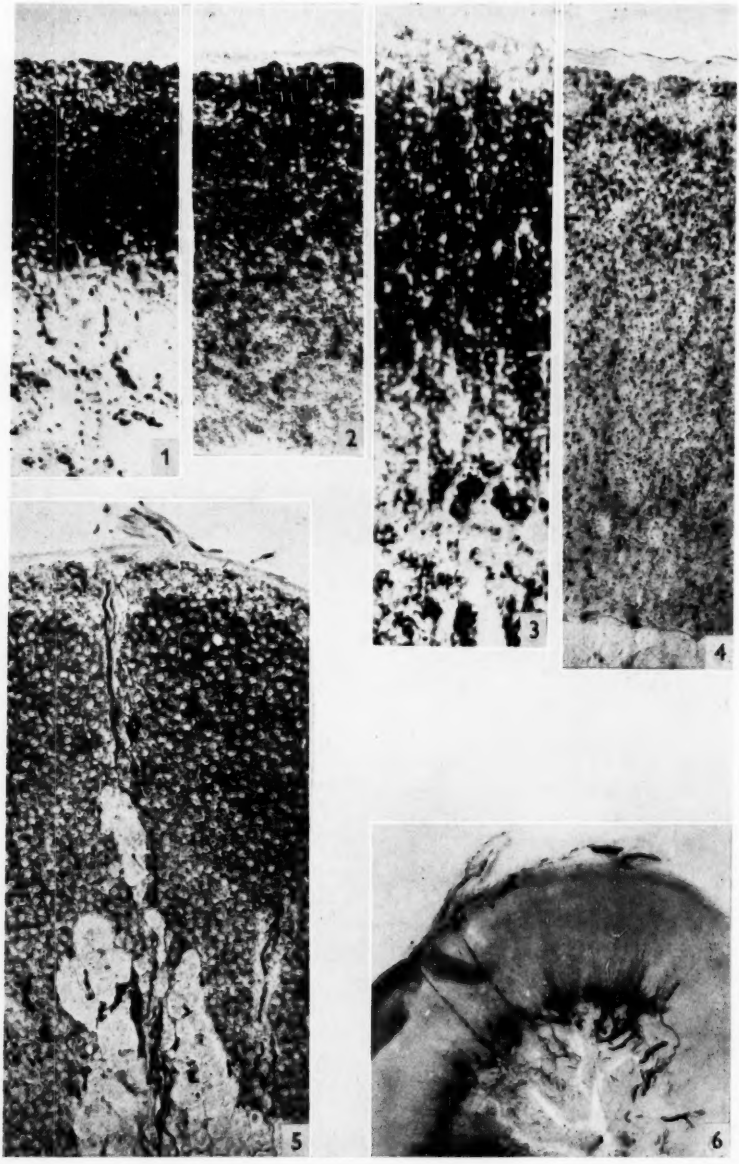

CAIN AND HARRISON—VARIATIONS IN THE ADRENAL CORTEX OF THE ALBINO BAT